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MOLECULAR DETERMINANTS OF ENDOCANNABINOID-MEDIATED BRAIN DEVELOPMENT

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KI-NIH Collaborative Doctoral Program in Neuroscience

Stockholm 2017

Cover artwork: *Brainbow*. Mirrored confocal image. Immunohistochemical double labelling with Hoechst and L1-NCAM antibody on organotypic E14.5 brain slice.

Published by Karolinska Institutet.
Printed by E-Print AB 2017

ISBN 978-91-7676-545-6

Molecular determinants of endocannabinoid-mediated brain development

THESIS FOR DOCTORAL DEGREE (Ph.D.)

ACCADEMIC DISSERTATION

The public defence of this thesis will take place at the Lecture Hall Cell and Molecular Biology (CMB), Berzelius väg 21, Karolinska Institutet, Stockholm, Sweden

Friday 31st of March 2017, 9.30 AM

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*“*Student: *Dr. Einstein, aren't these the same questions as last year's [physics] final exam?*

Dr. Einstein: *Yes; but this year the answers are different.”*

Attributed to Albert Einstein (1879, Ulm - 1955, Princeton)

ABSTRACT

The enchanting process of brain development entails a fragile and finite time window, throughout which biochemical imbalances and external insults can potentially affect developing neuronal circuits. Within the numerous exquisitely orchestrated events taking place in the foetal brain, axonal pathfinding is of paramount importance. If aberrant, axonal outgrowth can lead to errors in synaptogenesis and impaired connectivity, with potential long-lasting behavioural phenotypes. Despite the prominent role of endocannabinoids (eCBs) in axonal guidance, the molecular determinants of eCB-mediated axonal regulation remain largely unexplored.

Aiming to interrogate the eCB machinery in less explored subcortical territories, ***study I*** investigates the role of the eCB system and its underlying components in foetal cholinergic projection neurons. Prenatal manipulation of cannabinoid receptor 1 (CB_1R) permanently reshaped septo-hippocampal cholinergic projections. Nerve growth factor (NGF) was identified as an upstream molecule capable of regulating 2-arachydonoylglycerol (2-AG) via monoacylglycerol lipase (MAGL) degradation. The compartmentalization of each eCB machinery component along extending neurites is therefore crucial. However, axonal pathfinding takes place within the extracellular matrix, migrating glia and developing oligodendrocytes, generating a convoluted scenario where each growth cone interacts with multiple guidance proteins. Cannabinoid receptor 2 (CB_2R)-expressing oligodendrocytes are introduced in ***study II*** as an additional player able to drive aberrant callosal axons spread upon supra-physiological 2-AG levels. Excess 2-AG engaged CB_2R-mediated premature proliferation of oligodendrocyte end-feet. The interaction between the oligodendrocyte-derived chemorepellant molecule Slit2 and its receptor roundabout 1 (Robo1) on corticofugal axonal ends induced errant CB_1R-expressing corticofugal axon pathfinding. In addition to being an endogenous receptor of eCBs, CB_1R is also a target for the principal psychoactive compound of cannabis, Δ^9-tetrahydrocannabinol (THC). In ***study III*** we undertook an unbiased proteomics screening of the embryonic cortical plate following maternal THC exposure. Superior Cervical Ganglion 10 (SCG10), a microtubule-binding protein present in extending growth cones, was identified as a direct molecular target of THC. Thus, microtubule dynamics represent a novel target of prenatal THC exposure, whose impairment promotes axonal defects and long-lasting synaptic connectivity impairment. Cholecystokinin (Cck)-containing interneurons, due to their high expression of CB_1Rs and early appearance in the developing cortex, are likely targets of *in utero* eCB imbalances. Taking advantage of the recent $Cck^{BAC/DsRed}$ mouse line in ***study IV*** we achieved a systematic anatomical and physiological characterization of Cck-CB_1R-expressing cells from embryonic day (E)10.5 to adulthood. In sum, multiple upstream and downstream molecular components of the eCBs system were assessed over the course of this thesis investigation, within both physiological brain development and upon maternal cannabis exposure.

LIST OF SCIENTIFIC PAPERS

Study I. Nerve growth factor scales endocannabinoid signalling by regulating monoacylglycerol lipase turnover in developing cholinergic neurons. Keimpema E, Tortoriello G, Alpár A, Capsoni S, Arisi I, **Calvigioni D**, Hu SS, Cattaneo A, Doherty P, Mackie K, Harkany T. *PNAS*. 2013

Study II. Endocannabinoids modulate cortical development by configuring Slit2/Robo1 signalling. Alpár A, Tortoriello G, **Calvigioni D**, Niphakis MJ, Milenkovic I, Bakker J, Cameron GA, Hanics J, Morris CV, Fuzik J, Kovacs GG, Cravatt BF, Parnavelas JG, Andrews WD, Hurd YL, Keimpema E, Harkany T. *Nature Communication*. 2014

Study III. Miswiring the brain: Δ9-tetrahydrocannabinol disrupts cortical development by inducing an SCG10/stathmin-2 degradation pathway. Tortoriello G, Morris CV, Alpar A, Fuzik J, Shirran SL, **Calvigioni D**, Keimpema E, Botting CH, Reinecke K, Herdegen T, Courtney M, Hurd YL, Harkany T. *EMBO J*. 2014

Study IV. Functional differentiation of cholecystokinin-containing interneurons destined for the cerebral cortex. **Calvigioni D**, Máté Z, Fuzik J, Girach F, Zhang MD, Varro A, Beiersdorf J, Schwindling C, Yanagawa Y, Dockray GJ, McBain CJ, Hökfelt T, Szabó G, Keimpema E, Harkany T. *Cerebral Cortex*. 2016

PUBBLICATIONS NOT INCLUDED IN THE THESIS

Eps8 controls dendritic spine density and synaptic plasticity through its actin-capping activity. Menna E, Zambetti S, Morini R, Donzelli A, Disanza A, **Calvigioni D**, Braida D, Nicolini C, Orlando M, Fossati G, Cristina Regondi M, Pattini L, Frassoni C, Francolini M, Scita G, Sala M, Fahnestock M, Matteoli M. *EMBO J*. 2013

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The endocannabinoid 2-AG controls skeletal muscle cell differentiation *via* CB1 receptor-dependent inhibition of Kv7 channels. Iannotti FA, Silvestri C, Mazzarella E, Martella A, **Calvigioni D**, Piscitelli F, Ambrosino P, Petrosino S, Czifra G, Bíró T, Harkany T, Taglialatela M, Di Marzo V. *PNAS*. 2014

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A novel organizing principle of the hypothalamus reveals molecularly segregated periventricular dopamine neurons. Romanov R, Zeisel A, Bakker J, Girach F, Hellysaz A, Tomer R, Alpár A, Mulder J, Clotman F, Keimpema E, Hsueh B, Crow A, Martens H, Schwindling C, **Calvigioni D**, Bains J, Máté Z, Szabó G, Yanagawa Y, Zhang M, Rendeiro A, Farlik M, Uhlén M, Wulff P, Bock C, Broberger C, Deisseroth K, Hökfelt T, Linnarsson S, Horvath T, Harkany T. *Nature Neuroscience*. 2016

CONTENTS

LIST OF ABBREVIATIONS

2-AG	2-arachidonoylglycerol
ABHD	α/β domain-containing hydrolase
AEA	anandamide, arachidonoyl ethanolamine
ASD	autism spectrum disorders
BDNF	brain-derived neurotrophic factor
BRCA1	breast cancer 1 susceptibility protein
CAMs	cell adhesion molecules
CB_1R	cannabinoid receptor 1
CB_2R	cannabinoid receptor 2
CBD	cannabidiol
Cck	cholecystokinin
CGE	caudal ganglionic eminence
ChAT	choline acetyltransferase
CNPase	2',3'-cyclic-nucleotide 3'-phospodiesterase
CNS	central nervous system
COX-2	cyclooxygenase-2
CP	cortical plate
CR	calretinin
CRF/CRH	corticotropin-releasing factor
CSF	cerebrospinal fluid
DAG	diacylglycerol
DAGL	diacylglycerol lipase
DSE	depolarization-induced suppression of excitation
DSI	depolarization-induced suppression of inhibition
E-	embryonic day
eCB	endocannabinoid
ECMs	extracellular matrix components
ESC	embryonic stem cells
FAAH	fatty acid amide hydrolase
GABA	γ-aminobutyric acid
GAD67	glutamic acid decarboxylase 67
GDE-1	glicerophosphodiester phosphodiesterase I
GPCR	G-protein coupled receptor
HGF	hepatocyte growth factor
HPA	hypothalamus-pituitary-adrenal
Ig	immunoglobulin

IGF	insulin-like growth factor
IPC	intermediate progenitor cell
IPSC	induced pluripotent stem cells
iTRAQ	isobaric tagging for relative and absolute quantification
JNK1	c-Jun N-terminal Kinase 1
L1-NCAM	L1 neuronal cell adhesion molecule
LGE	lateral ganglionic eminence
LPI	lysophosphatidylinositol
LTD	long-term depression
MAGL	monoacylglycerol lipase
MAPs	microtubule-associated proteins
MGE	medial ganglionic eminence
mGluR	metabotropic glutamate receptor
MHPCD	Maternal Health Practices and Child Development Study
MS	medial septum
MSE	metabotropic-induced suppression of excitation
MSI	metabotropic-induced suppression of inhibition
MZ	marginal zone
NAPE	N-arachidonoyl phospatidyl ethanol
NAPE-PLD	NAPE-phosholipase D
NF-κB	nuclear factor Kappa B
NGF	nerve growth factor
NMII	non-muscle myosin
NSDUH	National Survey on Drug Use and Health
NT3	neurotrophin 3
NT4	neurotrophin 4
OPPS	Ottawa Prenatal Prospective Study
P-	postnatal day
$p75^{NTR}$	low-affinity neurotrophin receptor p75
pcd	post-conception day
pcw	post-conception week
PET	positron emission topography
PFC	prefrontal cortex
PI3K	phosphatidylinositol-3 kinase
PKC	protein kinase C
PLC-γ	phospholipase C gamma
PPARs	peroxisome proliferator activated receptors
PUFA	polyunsaturated fatty acids
PV	parvalbumin

RC2	radial glial cell marker-2
RG	radial glia
RMTW	rostro-medial telencephalic wall
Robo1	roundabout 1 receptor
ROCK	Rho-associated kinase
SCG10	Superior Cervical Ganglion 10, stathmin-2
sIPSCs	spontaneous postsynaptic inhibitory events
SP	subplate zone
SSI	slow self-inhibition
SVZ	subventricular zone
TCA	thalamocortical axons
TFs	transcription factors
THC	Δ^9-tetrahydrocannabinol
TMS	tangential migratory stream
TNF	tumor necrosis factor
Trk	tropomyosin-related kinase receptor
TRP	transient receptor potential channels
VAChT	vesicular acetylcholine transporter
VPA	valproic acid
VTA	ventral tegmental area
VZ	ventricular zone

1 INTRODUCTION

1.1 MILESTONES OF BRAIN DEVELOPMENT IN MICE AND MEN

Neuroscience, like other biological sciences, grounds its knowledge on experimental animal models. Thanks to an increasing economical commitment and to the exponential technical advancements made over the last 30 years, preclinical studies have led to a massive expansion of our understanding of the mechanisms and illnesses of the brain. Data derived from primates and humans remain the minority due to an ensemble of ethical, economical and technical reasons. This scenario will likely persist into the near future despite the recent development of *in vitro* human technologies, such as cerebral organoids [1, 2] and human neuronal cultures derived either by induced pluripotent stem cells (IPSC) or embryonic stem cells (ESC) [3]. Thus, the vast majority of our knowledge of brain function has relied heavily on model organisms, predominantly rodents due to the numerous genetic tools available. Despite the observation that the human brain exhibits architectural principles and neurodevelopmental processes that are often conserved across all mammals, interspecies differences [4] [5, 6] ultimately yield species-specific behaviours.

Humans have a prolonged development, with longer gestational time, as well as expanded childhood [7] and adolescence maturation [8]. Consequently, the human brain develops at a slower pace compared to mice and others primates, generating a wider time window vulnerable to genetic and environmental factors that contribute to shape cognitive capabilities and eventual neural circuit disorders [9] (Figure 1). Moreover, the developing central nervous system (CNS) possesses species-specific anatomical features, especially in the neocortex, such as the characteristic subplate region [10] and unique properties of its proliferative zone, neuronal stem cells and progenitors [11, 12].

The evolving nature of the brain has granted humans the privilege of higher cognitive function. However, this evolution has required adjustment and refinement of numerous neurodevelopmental features and their timing, resulting in a novel vulnerability to particular disorders, such as intellectual disabilities and psychiatric disorders. Limitations of experimental animal models in modelling neurodevelopmental disorders are therefore not surprising [13]. Thus, being aware of species-specific neurodevelopmental patterns is central when planning experiments and interpreting ensuing results.

The first section of this introduction will describe developmental milestones in the human CNS and report, when significant, differences that exist in mice. Developmental mechanisms will focus primarily on studies from the neocortex, mainly because of the higher number of comparative studies available.

1.1.1 Gross structures development

From gestation through adolescence, human brain development is a two decades-long process, spreading from embryonic progenitors' assembly until cell death and synaptic pruning in late adolescence [14]. Within this time a predicted final number of 86.1 billion neurons (adult male, [15]) find their correct locations and establish appropriate connections, generating an estimated 164 trillion synapses in the adult human neocortex [16].

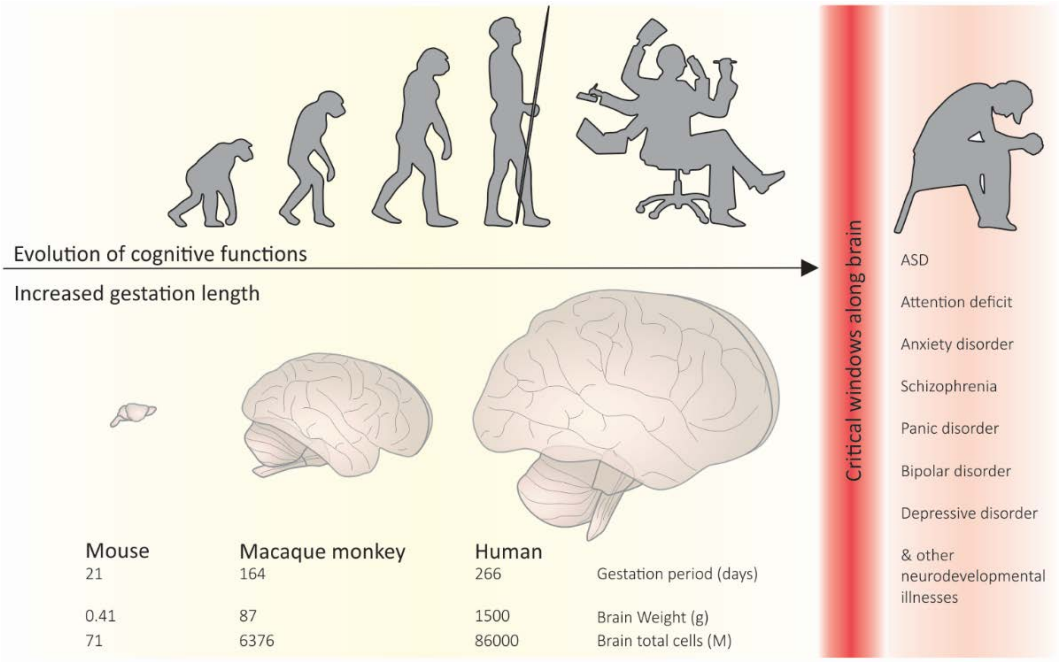

Figure 1. Evolution-driven increase in brain complexity. The evolving nature of the brain generated higher complexity of cognitive functions that characterizes humans. The required prolonged gestation contributed to generate critical windows along brain development and human-specific neurodevelopmental disorders. Gestational periods, brain weight and brain total cells are compared within mouse, macaque monkey and human. Data adapted from [15].

Human gestation typically lasts 38 weeks. The embryonic period begins at conception and extends through post conception week 8 (8 pcw), from which the foetal period begin (9 pcw till the end of gestation) [17]. By the end of the 3 pcw the embryo differentiates from a two layered structure into a three layered structure, a process known as gastrulation [18]. Neuroectodermal stem cells emerge during this phase and are capable of differentiating into any cell type present in the brain. The neuronal tube is formed during 3 pcw, when two ridges develop along the two sides of the neural plate, where neural progenitors lie [19]. During the next few days the ridges rise and close generating a cylindrical cavity, which eventually develops into the ventricular system of the brain [18]. By the end of the embryonic period five primary embryonic vesicles are formed along the rostral-caudal axis: the most anterior telencephalon and diencephalon, both derived from the prosencephalon, precursors of the forebrain; the middle mesencephalon, future midbrain; and the caudal metencephalon and myelencephalon, precursors of the hindbrain [17, 18]. Within the foetal period the human brain transforms from a smooth lissencephalic structure to a complex combination of gyri and sulci [18]. The longitudinal fissure starts caudally as early as 8 pcw, and it is completed in its rostral part at 22 pcw, separating the two cerebral hemispheres [20].

1.1.2 Neurogenesis and maturation of excitatory cells

Human neurogenesis starts as early as 4 pcw in the spinal cord and brain stem [18], and proceeds at location-specific rates during embryogenesis and foetal development with minor

postnatal contributions at defined locations. The majority of excitatory neurons are produced before 27 pcw [21] within the inner wall of the neural tube cavity, called the ventricular zone (VZ), and the adjacent subventricular zone (SVZ). Epithelial progenitors in the VZ differentiate into a population of stem cells, called radial glia (RG) progenitors [22]. The RG progenitor pool exponentially proliferates *via* symmetric cell division between 25 and 42 post conception days (pcd), resulting in a dramatic enlargement of the SVZ and VZ during early gestation. RG progenitors can divide symmetrically generating two daughter RG cells, or asymmetrically, yielding an intermediate progenitor cell (IPC) and a differentiated neuron [23]. Neurogenesis switches from a symmetrical to an asymmetrical mode around 50 pcd, when postmitotic neurons leave the proliferative zone and start to radially migrate in the developing neocortex in a subtype characteristic manner [18]. As discussed above, the timeframe of brain development in humans differs from other species, largely due to its complexity. This is best appreciated in the cortical neurogenesis timeframe, which is estimated to last ~143 days in humans (from 48 to 191 pcd), compared to 67 days in the macaque (from 45 to 112 pcd) and 11 days in mouse (from 11 to 21 pcd) [24] (see http://www.translatingtime.net/ for further neurodevelopment time differences across species). Despite the time differences, cortical glutamatergic pyramidal neurons are generated from RG progenitors of the VZ and migrate radially in the cortical plate (CP) in both humans and rodents [25].

Before glutamatergic cortical neurogenesis commences, a peculiar neuronal population develops, including reelin-expressing Cajal-Retzius cells and predecessor cells, and starts to migrate tangentially from outside the neocortex where they settle above the VZ, forming the outer marginal zone (MZ) [22]. MZ-resident early born cells have a central role to ensure correct cortical development, regulating laminar organization and early synaptic contacts of principal cells [26]. Below the MZ, the subplate zone (SP) gains tremendous importance during pregnancy. The SP prenatally expands to become the largest compartment of the developing cortex [10]. Recent studies highlighted the exceptional nature of the SP region (see below), revealing its transient nature, the heterogeneity of the population involved, an interesting set of human-specific features and the involvement of subplate neurons in developmental brain disorders [10]. During late foetal development, while SP neurons are reduced [10], CP neurons migrate to their final position in a precise inside-out manner [27], where they start to differentiate complex dendritic arbours and form local synaptic connections. Pyramidal cells develop basal and apical dendrites in the CP around 15 pcw [28], followed by the first synapse establishment around 18 pcw [29] and only later by dendritic spine development between 24 and 27 pcw [28]. Although starting during midgestation, synaptogenesis continues postnatally until the second year of life and peaks between 3rd and 15th month of life. Synaptogenesis is followed by a long period of synaptic reorganization, where extrinsic and intrinsic factors drive synaptic pruning [30]. Major synaptic elimination and dendritic contact reinforcement continues through childhood and adolescence [31], with both processes essential for maturation of cognitive functions.

1.1.3 The long migratory journey of interneurons

In contrast to the short radial journey of excitatory cells, human γ-aminobutyric acid (GABA)-expressing inhibitory neurons destined to populate the cortex are generated within a transient germinal zone in the ventral telencephalon and undergo a remarkable tangential migration prior to radial migration [11, 12]. Ventral ganglionic eminences are anatomically divided into medial, caudal, and lateral (MGE, LGE and CGE), and are conserved across multiple species including rodents [32]. From these structures, a multitude of interneuron subtypes are generated, whose identity and heterogeneity largely overlaps between higher primates and rodents [10, 33].

Enormous progress has been achieved through investigations using animal models elucidating the spatial and temporal dynamics of interneuron development [32, 34-40]. Recent studies on humans and nonhuman primates revealed novel interspecies differences, both at genetic [5, 41, 42] and developmental levels [10, 12]. For example, even though the precise magnitude and molecular mechanisms are still under debate, dorsal telencephalic VZ/SVZ progenitors are proposed as an additional source of GABAergic cortical interneurons in primates, an evolutionary novelty contributing to the elaboration of higher cognitive functions [12].

1.1.4 Glia and brain development

Once confined to supportive and protective functions, glial cells have more recently assumed a central role in brain development. Human oligodendrocytes and astrocytes are derived from the RG progenitor pool at midgestation [23], with a peak around birth and sustained development through the postnatal period [43]. Gliogenesis is therefore extremely protracted in humans compared to mice [44], suggesting glia possible involvement in the evolution of neocortical cognitive functions.

Cortical astrocytes proliferate after a first step of direct differentiation from RG cells [23, 45]. Human astrocytes proliferate and differentiate mostly postnatally, during the 3rd postnatal year [46]. A minor morphologically mature prenatal population was identified in the neocortex as early as 15 pcw [45]. Interestingly, the timing of differentiation of astroglial cells overlaps with synapse development in the neocortex, suggesting a potential primary role in synaptogenesis [47, 48], dendritic spine formation [49] and synaptic pruning [50].

Along the same line, the majority of oligodendrocytes are generated through the first 3 years of life, with a protracted region-specific postnatal maturation process up to the third decade of life [43]. Oligodendrocyte maturation in non-human primates, studied in chimpanzees, is instead mostly complete by adolescence [44]. During development and preceding myelination, neuronal interactions with oligodendrocyte precursors appear to have a significant functional role in regulating axon guidance and synaptic plasticity [51]. Moreover, oligodendrocyte precursors receive synaptic inputs from interneurons, generating a transient synaptic network [52] required for oligodendrocyte maturation. Once successfully differentiated, oligodendrocytes generate myelin sheaths around most axons, which are necessary for fast axonal conduction. Axonal myelin maturation inhibits cellular dynamism,

possibly leading to the end of the highly plastic synaptic rearrangement that characterizes adolescence. Interestingly, myelination and myelin dysfunctions seem to be highly specific for certain interneuron subclasses [53]. Inappropriate myelination of parvalbumin expressing interneurons (PV) was indeed proposed as a pathological locus responsible of PV interneurons functional impairment in schizophrenia [53].

1.1.5 The subplate: cells and development

Being one of the most diverse structures across different species, the subplate zone of the human brain is characterized by a transient dramatic expansion during pregnancy [54, 55]. The SP region plays critical roles in circuit assembly, being the intermediate target of long-range projection neurons and location of the first synaptic contact between migrating cortical neurons and ingrowing afferent fibres [55]. Populated by a heterogeneous population of 3.6 billion cells at its expansion peak around 31 pcw [56], the subplate zone is enriched in extracellular matrix, glial cells, migratory glutamatergic neurons, GABAergic neurons and cortical/subcortical axonal terminals [55, 57]. At 12-15 pcw the human subplate originates from the deeper cortical plate and becomes the dominant structure of the embryonic cortex [55]. The expansion in volume is characteristic of the “subplate stage” from 15 to 35 pcw, due to the massive ingrowth of axons invading the cortex, including thalamocortical afferents [58], forebrain cholinergic afferents [59], as well as contralateral and ipsilateral cortical projections [60]. From 35 pcw the human SP enters the final “dissolution stage” when its total volume shrinks due to a rapid decrease in the number of inhabiting cells, of extracellular space and axonal bundles [55]. Six months after birth the subplate is no longer visible in human tissue. In contrast, in rodents the subplate is not clearly identifiable by histological criteria and does not expand in volume during development [61]. The meaning of such diversity is at present unclear.

The origin of SP cells is heterogeneous, including both radially migrating excitatory cells originating from the SVZ together with ganglionic eminence-derived inhibitory neurons. The rostro-medial telencephalic wall (RMTW) generates an additional early born population of SP neurons [62]. RMTW-derived cells migrate dorsally to reach the cortex, spread through the subplate and secondarily colonize the nascent cortical plate by tangential migration, where they mature as Cajal-Retzius cells and deep-layers interneurons [62]. Even though only a few studies are available, interesting data have come from SP birth-dating experiments, both in primates and in mice [63]. Only early born neurons enter the SP and settle as part of the resident population, while cells born later do not take up residence in the SP, but rather migrate through [63]. Gene expression and ontology analyses have revealed early maturity as a characteristic hallmark of subplate neurons that are functionally enriched for axonal growth, cell adhesion, exocytosis as well as synaptic plasticity-related genes [64]. Moreover, recent transcriptome experiments in mice and humans have highlighted an expected human-specific pool of gene transcripts of the developing subplate, including genes involved in the production of extracellular matrix and proteoglycans, not present in the adjacent zones [65]. SP cells have in fact a distinctive early axonal protrusion, mature morphology and membrane

properties, compared to the cells populating the rest of the cortical plate, consistent with the earlier establishment of synaptic inputs to SP cells [66].

In addition to their diversity in origin, birthdate, and neurotransmitters, SP neurons are also morphologically heterogeneous during development [67] with fusiform, multipolar, polymorphic and inverted pyramidal shapes. At birth fusiform and polymorphic types are equally presents both in monkeys and humans [67]. In the adult, the morphology and function of the remaining cells is however less clear. With described species-specific variations, SP cells are the first cortical cells to send subcortical projections [68]. Both SP interneurons [69, 70] and excitatory cells [68] appear to form long range projections, the latter being responsible for ipsilateral cortical module maturation [61].

The cellular complexity of the SP zone mirrors multiple functional roles during development, highlighted by timed subplate ablation and misplacement experiments [71, 72]. Subplate neurons play critical roles early in development through thalamocortical axonal pathfinding, laminar positioning and synapse maturation, as well as in the establishment of sensory areas and oscillatory activity (for a comprehensive review on SP functions during development see [61]). Taken together the evidence indicates that the SP serves as a transient corridor for migrating excitatory and GABAergic neurons enriched in surface molecules, extracellular matrix and growth factors, where migratory switches and synaptic contacts take place. Subplate involvement in nervous system disorders such as epilepsy [73], schizophrenia [74] and autism [75] is therefore not surprising and worthy of further attention.

1.1.6 Critical windows along brain development

The emergence of human higher cognition, with its unique capabilities of abstract thinking and language, is the product of 300 million years of evolution of the mammalian brain [76]. The resultant complexity of the human CNS relies on a plethora of novel human-specific features, particularly evident in the neocortex. These include the introduction of new cytoarchitectonic areas, an increased complexity of the cortical columns, new genes and unique cell types [77]. The prolonged developmental time typical of the human brain described above is therefore an evolutionary necessity to achieve this higher complexity [14]. The trade-off for this two decades long maturation time [6] is however the opportunity for environmental and genetic risk factors to perturb or undermine the assembly of the physiological network. It is therefore not surprising that diseases in which human-specific behaviours are disturbed, such as intellectual disabilities, autism spectrum disorders and psychiatric disorders, happen to have a developmental origin [78].

The importance of intrauterine homeostasis has indeed been highlighted by numerous studies, causally linking neurological phenotypes to maternal infections, hypoxia, obesity and diet composition [79]. The idea of a perinatal non-genetic “maternal programming” consists therefore of maternal presentation of environmental or metabolic stimuli, specifically harmful because of their coincidence with critical windows during brain development [79]. The “double-hit” hypothesis was proposed for several neuropsychiatric disorders, where early perinatal environmental insults may lead to neuronal circuit vulnerability, while a

second “hit” during a later period, such as adolescence, may induce the onset of the disease [80, 81] (Figure 2). Hence maternal illicit drug use, inflammation and viral exposure during pregnancy have all been associated with cognitive/learning deficits and an increased risk of schizophrenia in offspring [82]. Moreover, delivery complications at birth [83] as well as stressor events during early development [84] positively correlate with psychiatric disorders. Furthermore longitudinal studies showed that behavioural phenotypes including intellectual disabilities incidence increases in offspring of obese mothers [85]. Revealing the signalling mechanisms connecting maternal inputs and offspring neurodevelopment, therefore, gains major significance.

Several milestones of brain development have been associated with GABAergic cells [78]. Although interneurons constitute only a minority of the total cortical neuron population (estimated to be ~15-20% in rodents [86]), they play crucial roles in network assembly, possibly explaining the involvement of GABAergic cells in a number of cognitive disorders [78]. In humans, as in rodents, GABA-mediated depolarization [87] provides the main source of synchronization [88] during embryonic and early postnatal time periods, which is required for correct activity-driven circuit development [89]. At birth, oxytocin drives a temporary switch of GABAergic signalling to inhibitory [90], by a reduction in intracellular chloride, which likely serves to protect against hypoxic stress during delivery. In rodents, the final maturation from excitation to inhibition for GABAergic transmission does not occur until the end of the first postnatal week [87], when the ultimate upregulation of chloride transporter expression in principal cells takes place. This postnatal switch of GABAergic signalling to providing inhibitory drive induces the maturation of interneuron-mediated fine-tuned responses that characterize human brain. This phenomena is known as desynchronization of the spontaneous network activity, and happens coincident with the initial sensory experiences, such as vision [91].

While less significant in adulthood, lack of correct stimuli exposure within critical windows of development have long-lasting effects [92]. Genetic and pharmacological experiments have highlighted GABAergic neurons as the principal gatekeepers of developmental critical windows [93]. Of the heterogeneous populations of inhibitory cells, PV-expressing fast-spiking interneurons are the major source of inhibition onto principal cells [94]. Moreover, PV interneuron maturation induces synchronous oscillatory activity in the neocortex, which is required for the establishment of functional connectivity between the hippocampus and the prefrontal cortex [95]. Interestingly, recent studies on schizophrenia pathophysiology have highlighted the central role of PV interneurons in disease onset [95, 96]. In general, multiple GABA receptors and overall inhibitory synapses are altered in schizophrenic patients [97] and, interestingly, in asymptomatic high-risk individuals [78].

To conclude, GABAergic interneurons play major roles in determining many of the critical windows of brain development, highlighting an interesting timed overlap between interneuron-mediated developmental events and neuropsychiatric disorder inception [98]. In addition to schizophrenia, interneurons are involved in the aetiology of a growing list of neuropsychiatric and developmental neural circuit disorders, including autism [99], epilepsy

[100], Rett's syndrome [101], fragile X syndrome [102], neurofibromatosis type I [103], Angelman's syndrome [104] and bipolar disorder [105]. Understanding how and when specific interneuron subtypes participate in each of these conditions will provide informed strategies for early therapies in high-risk individuals [98, 106], with a goal to not only treat symptoms but to prevent disease emergence.

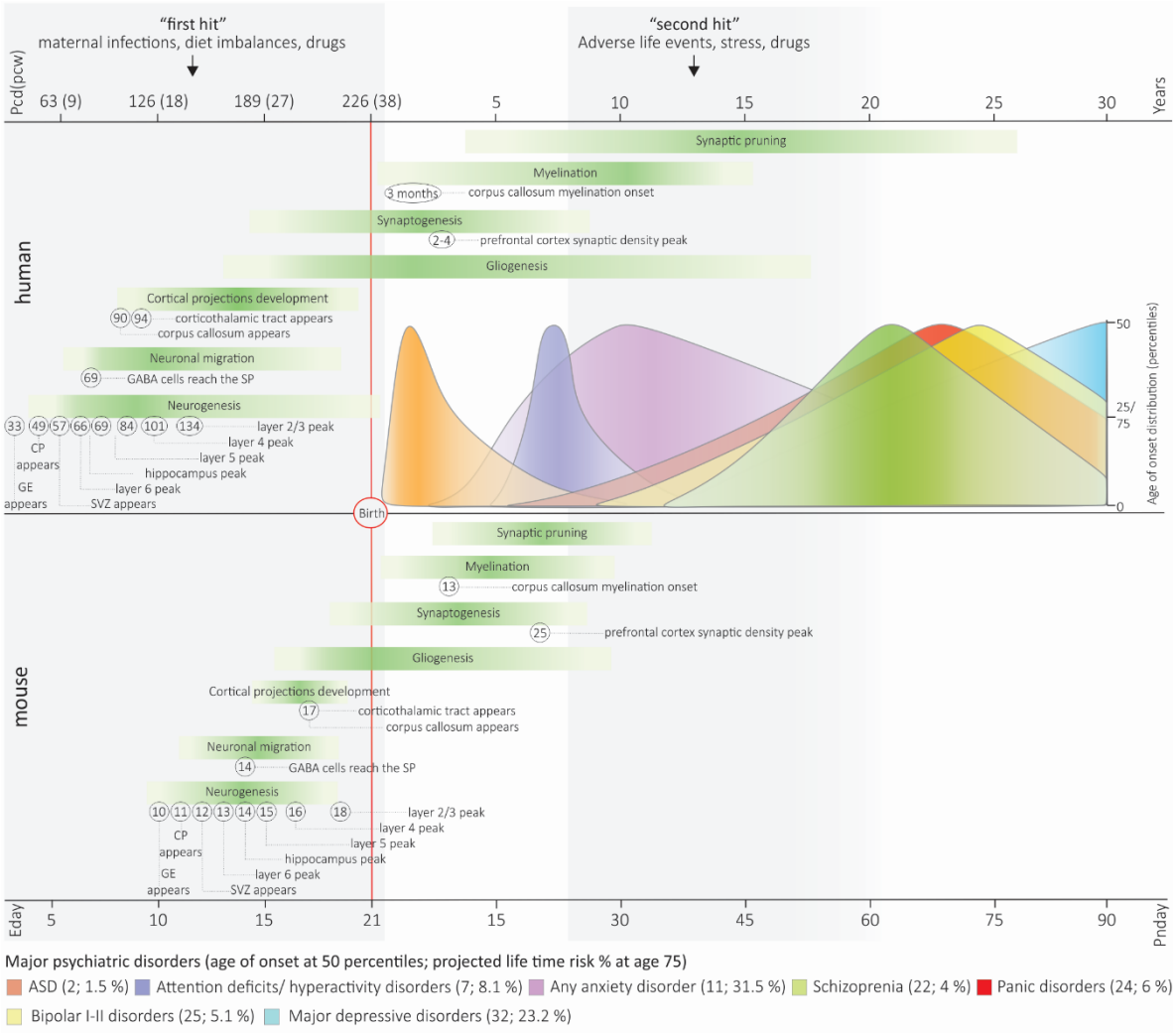

Figure 2. The "double-hit" hypothesis. Timeline of major neurodevelopmental events in human and mouse. Circles contain predicted timing of specific processes [24]. The age of onset of most prevalent neurodevelopmental disorders are represented as distribution curves [107]. The "first hit" consists of early perinatal insults, including maternal infections, diet imbalances and drugs abuse. Notice how the "first hit" coincides with key events during neurodevelopment (such as neurogenesis, migration, projections development and with the beginning of synaptogenesis and gliogenesis) therefore possibly leading to circuit vulnerabilities. A "second hit" later in time, possibly represented by drug abuse or adverse life events and stress during adolescence, may induce disease onset. The "second hit" overlaps with critical events for network maturation (such as synaptic pruning, myelination, and gliogenesis).

1.2 CELLULAR AND MOLECULAR LANDSCAPES OF AXONAL PATHFINDING

All developing neurons are required to sense the surrounding environment in order to target their correct postsynaptic partners. The sensory tips of each axonal projection, the growth cones, are highly motile and dynamic structures able to perceive the environment and address axons to their targets through the correct migratory path. Santiago Ramon y Cajal, who first described the growth cone structure in 1892, became aware of its nature using only silver-impregnation and his devoted observation skills:

> *"From the functional point of view the growth cone may be regarded as a sort of club or battering ram, endowed with exquisite chemical sensitivity, with rapid amoeboid movements, and with certain impulsive force, thanks to which it is able to proceed forward and overcome obstacles met in its way, forcing cellular interstices until it arrives at its destination."* [108]

Of all his observations, Cajal was certainly intrigued by the "intelligent force" driving the growth cone and its capability of chemical attraction by substances produced by the target structures, a phenomenon later named "chemotropism":

> *"How does the mechanical development of the nerve fibers occur, and wherein lies that marvellous power which enables the nerve fibers from very distant cells to make contact directly with certain other nerve cells or the mesoderm or ectoderm without going astray or taking a roundabout course. [...] If a chemotaxic sensitivity in the neuroblasts is assumed, then it must be supposed that these cells are capable of amoeboid movement and are responsive to certain substances secreted by cells of the epithelium or mesoderm [109]. The processes of the neuroblasts become oriented by chemical stimulation, and move toward the secretion products of certain cells."* [110]

Almost a century later the netrin family was identified in the developing axons of the spinal cord [111], representing first described chemoattractant cue and ultimately supporting Cajal's intuitions. Two decades later, the first live imaging of a growth cone [112] revealed its dynamic nature, a key feature responsible of the "intelligent" behaviour observed by Cajal.

1.2.1 Cytoskeletal dynamics of the growth cone

Macroscopically the growth cone appears as an enlarged terminal, present at the end of each axon and dendrite. The peripheral region (P region) of the growth cone is a flat area characterized by membrane protrusions named either lamellipodia, if enlarged and supported by a meshwork of branched actin filaments (F-actin), or filopodia when structurally maintained by long parallel bundles of actin linear arrays [109] (Figure 3). Extension of the lamellipodia and filopodia is a major dynamic event characterizing the peripheral region and is crucial for growth cone movement. The backbone of the dynamic tip of each axon, F-actin,

is the molecular target for numerous signalling cascades, and is regulated by a wide range of proteins involved in actin assembly/disassembly, crosslinking, end capping and monomer sequestering [113]. Extensive literature can be found for each of these families, including WASp [114], Arp2/3 [115], capping proteins [116, 117], ADF/cofilin [118] and thymosin/profilin [119]. Optimal regulation of actin-associated proteins is critical for the high active turnover typical of explorative young growth cones and their sequential switch into mature neurons, characterized by relatively stable F-actin (for extensive reviews on cytoskeleton dynamics see [113, 120, 121]).

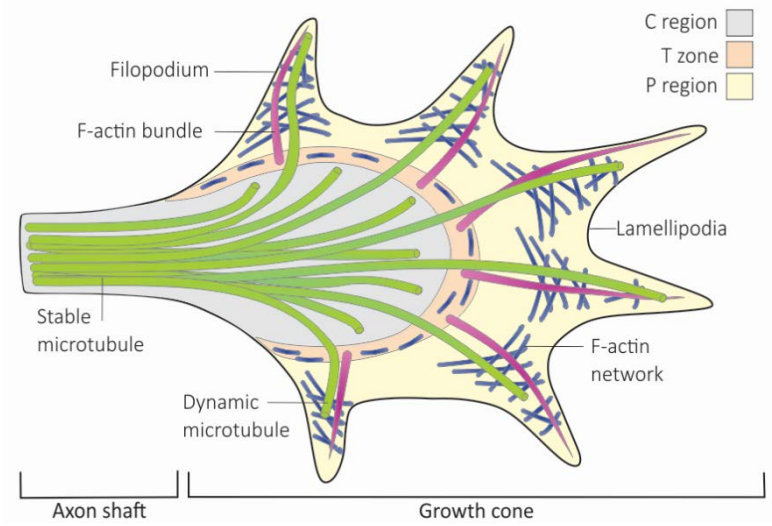

Figure 3. The growth cone and its cytoskeleton. The tip of the growth cone can be divided in three structural regions, distinct in function and cytoskeletal components. The C region (*grey*) connects the growth cone to the axonal shaft and contains the majority of microtubules (*green*). The T zone (*orange*) is the intermediate zone that controls growth cone motility and contains actomyosin contractile structures known as actin arcs (*blue*). The outer region, or P zone (*yellow*) is the motile and sensory region of the axon. It is characterised by enlarged lamellipodia supported by branched F-actin networks (*blue*) and filopodia, containing F-actin bundles (*magenta*), F-actin networks (*blue*) and pioneer microtubules (*green*). Modified from *Lowery et al* [109].

Central to the outer structure is the body of the growth cone (C region), which is connected to the axonal shaft, and supports the movement of growth cone tips and contains mitochondria and exocytotic vesicles [109]. Structurally the C region is characterized by microtubules, a minority of which extend to an intermediate region between the body and the outer zone, known as the transitional zone (T zone), where actomyosin contractile structures (actin arcs) control growth cone motility [122]. Microtubules are polar structures comprising heterodimers of α and β subunits, each of which consists of 13 protofilaments [123]. The polarity of microtubules results in one fast-growing end named the “plus end” pointing the P zone and the opposite, slow-growing, “minus end” facing the C region [124]. The minus end is often capped and sequestered at the body of the growth cone [125]. At the

distal region of the neurite the plus end of individual microtubules extends to reach the actin-rich peripheral domain. Here a complex and continuous series of growing and shortening events take place, a process called dynamic instability [126]. Early pharmacology experiments revealed that the dynamic behaviour of microtubules is responsible and necessary to achieve axonal extension [126]. Cytoskeleton dynamic instability is therefore a key feature of the growth cone, necessary for its ability to quickly remodel upon extracellular signals.

Dynamic instability is regulated by two principal categories of molecules: microtubule stabilizer and destabilizing proteins [127]. Microtubule-associated proteins (MAPs) belong to the first category [128]. Strategies adopted to stabilize microtubules include increasing the frequency of dimers rescue, binding the microtubule polymer, and bundle formation upon microtubule crosslinking [121]. Amongst microtubule destabilizers, the stathmin family has been identified as intracellular factors that promote microtubule depolymerisation by likely increasing the rate of "catastrophe" events [129-131].

1.2.1.1 SCG10 controls microtubule dynamics via JNK1

Amongst stathmins, a family of ubiquitously expressed proteins involved in the physiological regulation of microtubule dynamics [132, 133], the neuron specific member SCG10 (also named brain-specific stathmin-2) was initially identified as a neuronal marker in the neural crest [134]. SCG10 expression is neuron-specific and finely upregulated during development [129]. SCG10 is richly localized to outgrowing neurites both in the developing retina [135] and in cortical neurons [129], and is strongly downregulated after synapse formation [129]. Moreover, in the rat olfactory system SCG10 expression is restricted to immature olfactory neurons, and is highly upregulated after injury during olfactory axon regeneration [136]. Although the molecular cascade(s) involved is not yet fully understood, early studies suggest a principal role for SCG10 in axonal development and regeneration.

One of the first studies aimed at dissecting the intracellular cascade downstream of stathmin pointed out that SCG10 overexpression *in vitro* inhibits microtubule polymerization in a dose dependent manner [137]. The mechanism by which this happens is similar to stathmins' ability to sequester tubulin molecules [138]. SCG10 binds tubulin dimers, generating a ternary complex ("T2S") of one SCG10 molecule and two α/β tubulin dimers [139] (Figure 4). Tubulin sequestration lowers the amount of intracellular available soluble tubulin dimers, resulting in an overall inhibition of the polymerization of microtubule filaments [140] and an increase in catastrophe events. SCG10 also acts at the minus end of microtubules where it mediates instability by increasing the shortening rate [141].

Cellular levels of SCG10 are regulated by post-translational modifications such as phosphorylation [133]. Mass spectrometry revealed four phosphorylation sites on SCG10: Ser50, Ser97, Ser62 and Ser73 [142]. SCG10 phosphorylation by c-Jun N-terminal Kinase 1 (JNK1), the brain specific isoform of

the JNK family [143], was described as a neuronal specific pathway in axonal degeneration [144] and multipolar stage exit during neuronal migration [145]. JNK1 recruitment phosphorylates SCG10 specifically on Ser62 and Ser73, inducing SCG10 inactivity and

proteasomal degradation [144]. JNK1 was already introduced as an intracellular component involved in extracellular axon guidance [143]. JNK-mediated SCG10 degradation is therefore mechanistically appealing as a rapid on-demand cellular mechanism for regulating cytoskeleton dynamics in response to extracellular guidance molecules. However, the upstream extracellular signals capable of inducing this intracellular pathway remain to be explored.

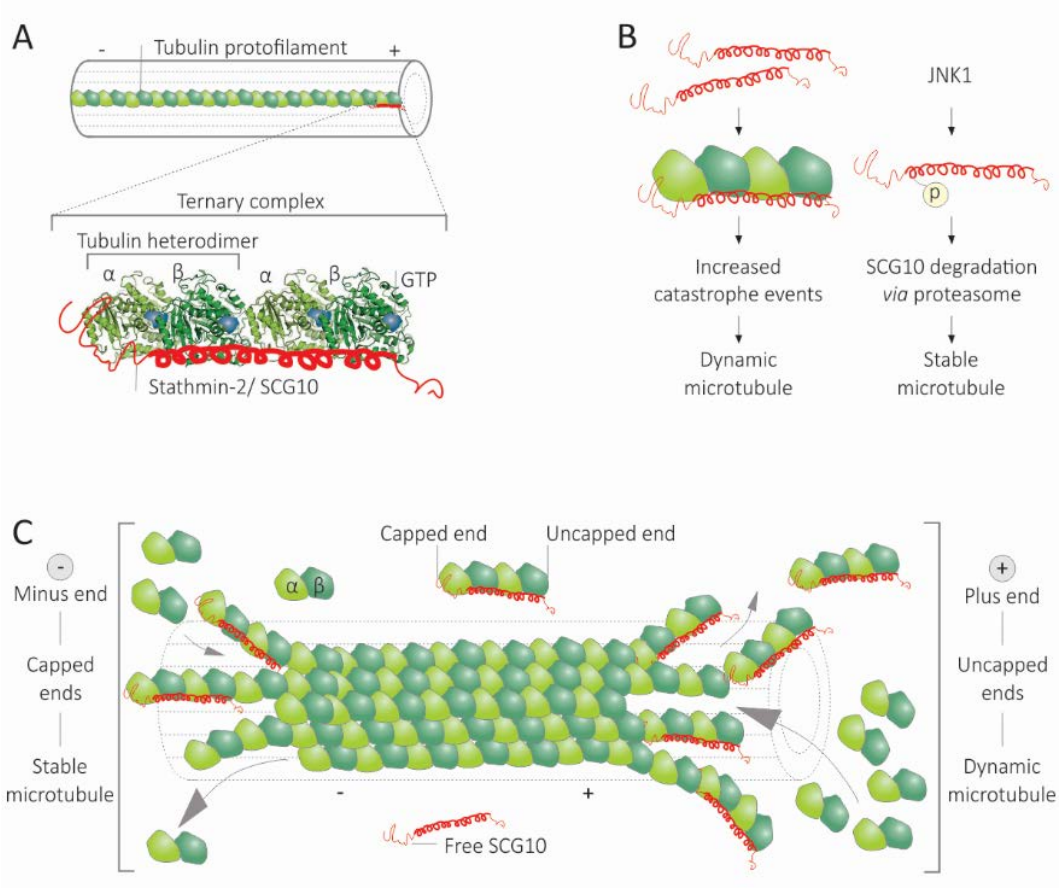

Figure 4. SCG10 controls microtubule dynamic instability. (A) Microtubules are cylindrical polar structures consisting of tubulin protofilaments. Each tubulin heterodimer consists of α (*light green*) and β subunits (*dark green*), each tightly bound to its GTP molecule (*blue*). Stathmin-2/SCG10 (*red*) binds to two tubulin heterodimers, forming a ternary complex. Adapted from *Essential cell biology, Garland science* [146]. **(B)** SCG10 availability controls microtubule dynamism. SCG10 forms ternary complexes with tubulin heterodimers at the plus end of each protofilament, promoting catastrophe events and microtubules instability. JNK1 leads to SCG10 degradation *via* proteasome after inducing SCG10 phosphorylation. SCG10 reduced availability results in a more stable microtubule [144]. **(C)** Schematic illustration of SCG10-mediated catastrophe events. SCG10 binds at the minus end of the microtubule, capping the exposed α-tubulin and prevents the incorporation of new dimers, thus stabilizing the minus end. At the plus end SCG10 promotes instead catastrophe events by multiple mechanisms: inhibiting lateral interactions between protofilaments, inducing curvatures and increasing GTPase activity at the uncapped ends, overall destabilizing the tip and mediating catastrophe events. In sum, SCG10 contributes to generate microtubule asymmetric catastrophe events at the two polar ends. Adapted from *Gupta et al* [147].

1.2.2 Molecular guidance cues orchestrate axonal outgrowth

The intrinsic ability of growth cones to sense their surroundings implies an evolutionarily conserved preference of specific axons for certain migratory pathways. Migrating cells are indeed able to sense a wide range of guidance cues, including localized molecular cues [148], chemical [149] and electrical gradients [150] as well as mechanical signals [151]. While the latter have only started to be recently explored, from Cajal onwards, increasing efforts have been invested in identifying and characterizing the molecular cues involved in the extension of the axonal tip. Because of the large number of existing guidance cues, their expression must be finely regulated in order to reproduce the correct pattern of axonal outgrowth [152]. The expression patterns of guidance cues segregate between subcellular structures and cell types [152]. Although we focus here on the guidance of primary axonal growth cones, the extending neurite often branches forming secondary growth cones [153]. Collateral sprouts are initiated and directed by the same guidance cues as the primary growth cone [153].

Accepting the limit of categorization in specific contexts, individual molecular guidance cues fall into three main classes: adhesive cues, trophic signals and chemotropic guidance cues. Adhesive cues include extracellular matrix components (ECMs) and cell adhesion molecules (CAMs), both generally recognized by specific receptors on the growth cone [154] (Figure 5). ECM subtype distributions, including those for laminin, collagen, fibronectin and a variety of proteoglycans, define a substrate as permissive or non-permissive [154]. ECM receptors are typically part of the Integrin family [155]. Notably neurons are able to express their preference on specific ECMs depending on the type of Integrin receptor expressed [156]. A combinatorial variety of CAMs are expressed not only in neurons but also in glial cells [157]. Immunoglobulin (Ig) and calcium-dependent cadherin represent the two major families of CAMs [158]. Members these superfamilies mediate axonal guidance either *via* heterophilic interaction or through homophilic adhesion, acting as ligand or receptor in opposing cells [159]. More than 50 neuronal CAMs are encoded in mammals, each with specific interactive extracellular domains and intracellular signalling cascades [158].

Trophic signals are by definition molecules that promote cell survival. However additional roles in axonal outgrowth have been highlighted for numerous trophic signals, including neurotrophins [160], insulin-like growth factor (IGF) [161] and hepatocyte growth factor (HGF) [162]. Mammalian neurotrophins are a heterogeneous family of small molecules that include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3) and neurotrophin 4 (NT4). Neurotrophins activate a multi-receptor signalling network that triggers a variety of complex intracellular cascades, driving a plethora of cellular events, including cell survival, cell death, promotion and inhibition of neurite outgrowth [160] (for an extensive review on the intracellular cascades engaged by neurotrophins see [160]). Two principal receptor types bind mature neurotrophins: 1) the low affinity $p75^{NTR}$, a member of the tumor necrosis factor (TNF) superfamily; and 2) the high affinity tropomyosin-related kinase receptor (Trk) family [163, 164]. Each neurotrophin family member selectively binds Trk receptors: NGF binds to TrkA; BDNF and NT4 bind to TrkB; and NT3 binds to TrkC; all neurotrophins bind to $p75^{NTR}$ [163, 164]. Exceptions to this rule, together with regulatory

intermediates within each intracellular signalling cascade, highlight neurotrophins as one of the most finely regulated guidance mechanisms.

Chemotropic guidance cues are often secreted molecules that establish attractant or repellent gradients, able to impart directionality from a distance on the growing axon. Alternatively, guidance cues can be transmembrane or surface bound molecules, and influence growth cones only locally [154]. Of the first type, netrins are a small family of diffusible molecules with an interesting bifunctional role capable of repelling some axons while attracting others [165]. Slit family, secreted but exclusively chemorepellant, bind Robo1/2 receptors and control the guidance of the major axonal tracts and interneuron migration in the developing brain [166]. The large family of Semaphorins includes both secreted and transmembrane chemorepellent proteins [167]. Netrins, Semaphorins and Slits, each act through their own receptor families to achieve specific guidance outcomes by regulating the activity of Rho GTPases, which in turn modulate cytoskeleton dynamics [148].

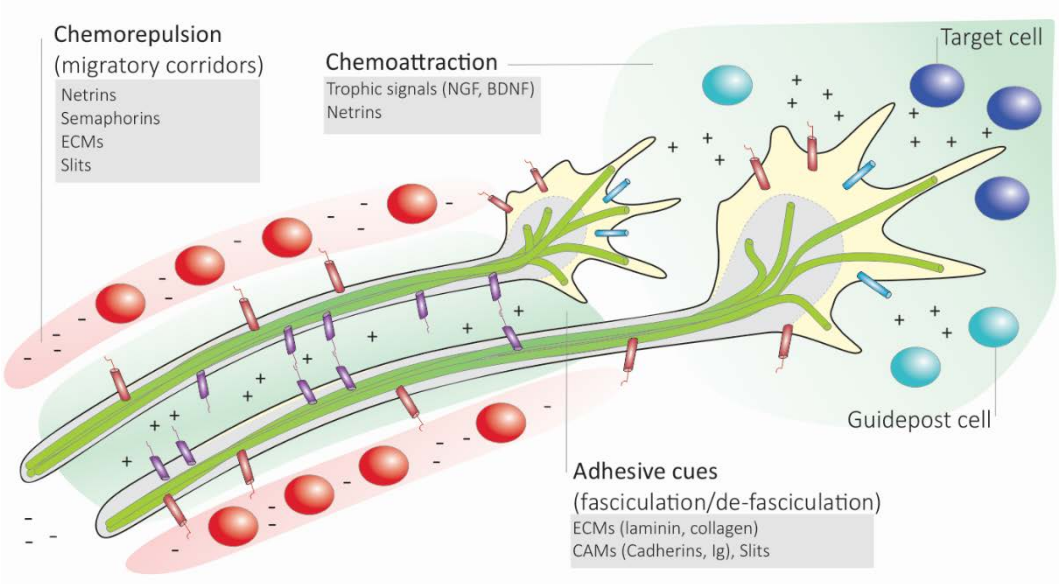

Figure 5. Molecular guidance cues drive axonal outgrowth. Axonal outgrowth takes place within numerous chemoattractive (+) and chemorepulsive cues (-) that generate migratory permissive roads (*green background*) and surrounding repulsive guard rails (*red background*). Chemoattractive cues induce filopodia protrusion by F-actin bundle polymerization and exploratory microtubule recruitment at the tip of the P zone (*yellow*). When the growth cone faces repulsive cues lamellipodia structures prevail on the growth cone and microtubules are restricted to the C zone (*grey*). Repulsive cues generated by cells at critical positions (*red circles*) generate repulsive corridors able to restrict permissive territories. Chermorepulsive cues can be transmembrane or secreted and impart directionality on certain neurons due to specific receptor expression patterns (*red*). Along the migratory path, axon fasciculation and de-fasciculation events are orchestrated by short-range adhesive cues and their receptors (*purple*). Intermediate guidepost cells (*cyan circles*), such as glia or subplate cells, contribute to growth cone guidance towards final targets (*blue circles*). Both target neurons and intermediate guidepost cells attract migratory neurons generating a gradient of long-range positive cues sensed by growth cone tips using specific receptors (*blue*).

1.2.2.1 NGF regulates cholinergic projection development in the basal forebrain

The first neurotrophin to be isolated was NGF, in 1951, by the Italian scientist Rita Levi-Montalcini [168], recognized for her scientific contribution with the Nobel Prize in 1986. The identification of NGF changed the perception of cellular communication confirming that cells can target distant organs and communicate by secreted factors.

NGF is a glycoprotein of 118 amino acids, synthesized as pro-NGF and cleaved either intracellularly or after secretion into mature NGF [169]. Once intracellularly synthesized the N-terminal sequence of NGF mediates translocation to the endoplasmic reticulum and packaging into vesicles before secretion [169]. While the first understood function of neurotrophins was cell survival, it soon became apparent that NGF promotes axonal growth driving F-actin polymerization in the growth cones and during filopodia formation of sensory neurons [169]. NGF is indeed synthesized from target tissues and once secreted interacts with its receptors at axonal ends [170]. Even if no data were available at the time, Rita Levi-Montalcini foresaw NGF chemotactic effects in neurodevelopment. Her words at the Nobel Price lecture in 1986 are a testament to her scientific instincts and enthusiasm:

> *"The most obvious among the foreseeable approaches is the search for other NGF target cells, using the ever more sophisticated in vivo and in vitro techniques which became available in these last decades. It was this multimodal approach, which in recent years led to the discovery of NGF target cells in the CNS of lower and higher vertebrates and in cell lineages playing a role in the immune system. This list is likely to increase, as the search extends to other neuronal and non-neuronal cell populations. Furthermore, one should take into account the fact that some of these populations are receptive to NGF mainly during developmental stages in prenatal life. […] These should provide valuable information on some of the still unexplored, submerged areas of the NGF iceberg, dealing with the processes of the NGF gene transcription or translation"* [171].

The chemotactic effects of NGF were indeed first described by the Levi-Montalcini laboratory in neonatal rats when they observed that an *in vivo* administration of NGF recruited massive growth of fibres towards the NGF source [170]. Subsequent efforts directed at understanding the molecular mechanisms mediating NGF actions on axonal growth cones, revealed TrkA receptors as the principal players involved in sensing NGF [172]. Once activated, TrkA receptors exhibit intrinsic tyrosine kinase activity promoting receptor autophosphorylation and subsequent activation of multiple intracellular signalling cascades [172] (Figure 6). Early studies on PC12 cells [173] revealed that neurite outgrowth is regulated *via* TrkA through three main enzymes, protein kinase C (PKC), phosphatidylinositol-3 kinase (PI3K) and phospholipase C gamma (PLC-γ), all acting downstream on actin [174]. Furthermore, these studies showed that neurite elongation and actin redistribution in NGF exposed PC12 cells are mediated by decreased phosphorylation of ADF/cofilin [175].

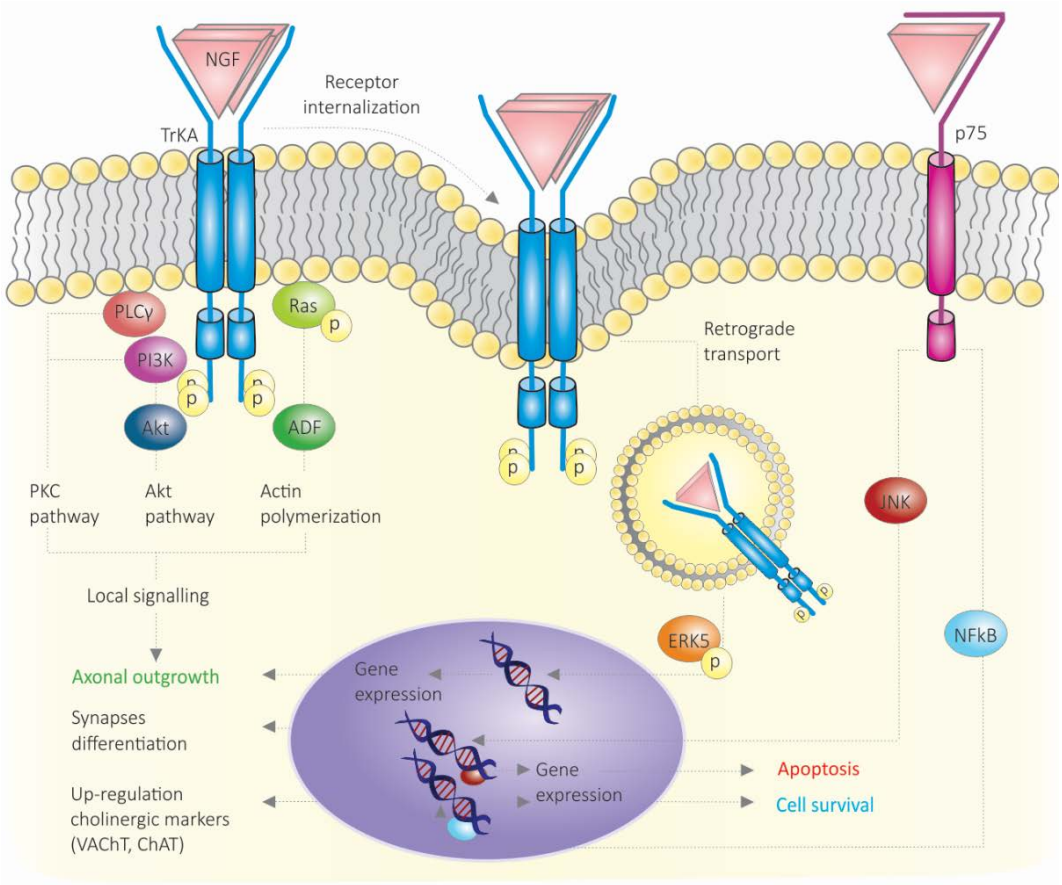

Figure 6. Nerve growth factor (NGF) intracellular signalling. NGF mediates neuronal growth and cell survival *via* the high affinity receptor TrkA and the low affinity receptor p75. TrkA engagement causes TrkA receptor dimerization and autophosphorylation, followed by a variety of local downstream signalling cascades. TrkA activation-mediated local signalling includes the recruitment of PKC pathway *via* PLCγ, Akt pathway *via* PI3K, and cytoskeleton reorganization leading to axonal outgrowth (for an extensive description of NGF-mediated intracellular cascade see [163]). Activated TrkA receptors within axonal terminals are internalized into vesicles and transported to the soma. In the soma ERK5 is activated and transported to the nucleus where it regulates gene expression. TrkA-mediated gene regulation includes mediators of axonal outgrowth, synapse differentiation and cholinergic markers. NGF induces cell survival *via* low affinity receptor p75 and NF-κB mediated gene regulation, while JNK-mediated cell death may be promoted by receptor p75 upon limiting amounts of NGF. Modified from https://www.qiagen.com/us/shop/genes-and-pathways/pathway-details/?pwid=456.

The roles of NGF in axon outgrowth, survival, and repair were extensively studied in long-range projections of basal forebrain cholinergic neurons targeting the cortex and hippocampus. Basal forebrain-hippocampal cholinergic projections have a central role in memory and attention processes [176] and are found to be dysfunctional in Alzheimer's disease [177]. Recent studies aiming to improve cholinergic cell survival in neurodegenerative diseases have, therefore, focused on neurotrophins, particularly NGF [178]. NGF synthesized in the cortex and hippocampus regulates development [179], cell size and dendritic arborisation of basal cholinergic neurons [180]. In the hippocampus NGF is produced by pyramidal neurons [181], glial cells [182], dentate granule neurons and subpopulations of GABAergic interneurons [183]. Though details of regulated NGF production remain to be

addressed, evidence indicates that NGF expression in the hippocampus is activity dependent [184], and markedly dysregulated under pathological conditions such as seizure, injury [185] and inflammation [182]. Once NGF binds to its receptor it has to be internalized and retrogradely transported along the axon towards the cytoplasm and the cell nucleus. This was initially revealed by retrograde accumulation of ^{125}I-labelled NGF in basal forebrain cholinergic cells following hippocampal injections [186]. Moreover, septo-hippocampal lesion results in atrophy of the cholinergic cells and over-accumulation of NGF in the hippocampal region [186]. These were the original observations that led to the hypothesis of a retrograde transport of NGF from the growth cone to the nucleus where gene expression could be consequently regulated [187]. The multiple pathways through which NGF transduces neurotrophic signals into gene expression are today mostly described, including the above cited PI3K, PLC-γ as well as mediators of survival and apoptosis such as Jun Kinase (JNK) and Nuclear factor Kappa B (NF-κB) [160]. Phenotypic knockout of NGF revealed that basal forebrain and hippocampal cholinergic neurons survival are particularly sensitive to NGF [188]. Additionally, NGF promotes the cholinergic neuronal phenotype by driving expression of cholinergic markers such as choline acetyltransferase (ChAT) [189] and vesicular acetylcholine transporter (VAChT) [190] with consequent regulation of acetylcholine synthesis and release [191]. Within the cholinergic system endogenous NGF is critical for cortical synapse differentiation and maintenance, as demonstrated by studies using anti-NGF monoclonal antibodies, TrkA receptor antagonists and TrkA knockout animals [192]. However, potential coordination between NGF and other secondary molecular effectors highly co-expressed in basal forebrain cholinergic projections, such as endocannabinoids [193], remains to be investigated.

1.2.2.2 Slit/Robo interactions modulate axonal pathfinding of major axonal tracts

Slit/Robo signalling was one of the first chemotropic guidance cue programs discovered [194]. First identified in *Drosophila*, where they control midline crossing of commissural axons [195], Slit/Robo interactions have central roles in the guidance of thalamocortical axons (TCA), cortico-cortical projections through the corpus callosum and corticofugal projections [196, 197].

TCA outgrowth from the diencephalon into the ventral telencephalon is initiated at E12.5 in mouse [198]. TCA fibers cross the striatum and enter the cortical plate by E14.5 [198]. Along their journey TCAs are guided by numerous guidance cues, such as neuregulins and guidepost cells, which promote extension and correct directionality of TCAs [199]. Numerous studies have defined a pivotal role for Slit/Robo signalling in TCA targeting [196, 200, 201]. Various combinations of Slit1/2 and Robo knockout mice exhibit thalamocortical targeting defects during their developmental trajectory [196, 202], and Robo1$^{-/-}$ mice have enlarged TCA bundles [203]. Moreover, Robo expression is regulated by Lhx2 and Gbx2 transcription factors (TFs) [204] [205] and both Lhx2 and Gbx2 mutant mice display defective TCA targeting [204, 205].

Slit/Robo signalling is critical for corpus callosum development and both Robo1/2 and Slit2 mutants present abnormal callosal projections [196]. A recent set of interesting studies

proposed that Slit1/3 controls callosal projections indirectly by regulating the glial population resident in this region [206], supporting the hypothesis that glial-neuron interactions direct axonal guidance. In glial cells, as in neurons, TFs regulate Slit2 expression [207]. Furthermore, glial expression of Slit2 is transcriptionally regulated by Gli3, which if absent perturbs corpus callosum development and relocates $Slit2^{+}$ midline glial cells [207].

In 20 years of research Slit/Robo signalling has extended well beyond its classical role in midline crossing and axonal pathfinding [196], being implicated in the migration of cortical neurons [208, 209], spine formation [210], dendrite development [211, 212] and cortical progenitor proliferation [213]. Despite numerous extensive studies on axonal tract development [194, 196, 197, 206, 214, 215], Slit/Robo signalling cascades and cellular functions remain incompletely understood. Moreover, while appealing, the hypothesis of axon-glia joint control over axonal outgrowth *via* Slit/Robo signalling [206, 207] requires further investigation.

1.2.3 Axon-glia interactions in neuronal pathfinding

Recent literature has moved beyond a model where axonal outgrowth is driven strictly by intrinsic developmental programs residing exclusively within the extending neurite. Rather, models proposing an interactive multiplayer scenario critically dependent on glia-neuron interactions, especially in early stages of development, are gaining popularity [216].

Glial cells act as guideposts, located at crucial decision points along migratory routes, towards which cells are attracted and further directed to their final target structure [217]. As cited above, this role was initially described at the midline for regulation of ipsilateral/contralateral projections. In this case, glia provided chemorepellant cues, secreting Slit at the midline [214, 218]. The timed expression of Robo receptors in ipsilateral and contralateral axons, either after or before they crossed the midline, was identified as a key mechanism [219, 220].

Once outgrowing axons reach their target area, growth cones receive specific targeting cues that stop their migratory behaviour and help them to identify correct postsynaptic partners. At this stage neurons, as well as glia cells, can act as intermediate temporary target cells. The role of glia as intermediate targets has been described for layer selection of retinal axons in Drosophila [221]. In vertebrates, the most described intermediate target cells are Cajal-Retzius cells [222] and subplate neurons [223] described above. Despite limited information about non-neuronal intermediate target cells in vertebrates, glia have been proposed to fulfil such a role in the rodent olfactory bulb, where olfactory axons contact glia tufted processes during glomerular formation [224]. Intriguingly, glia have been shown to interact with axons also at later stages, as shown for axonal pruning of mushroom bodies in Drosophila [225] and during synaptic refinement [226]. Taken together, the emerging idea of glia and neurons as team players in axonal guidance is an interesting and challenging new concept that needs further investigation.

1.2.4 Complex decisions along the migratory journey: axonal fasciculation

The guidance molecules and intracellular signalling cascades described separately *in vivo* take place often simultaneously, requiring well-orchestrated control over complex decision points. This is exemplified by guidance to and from the midline as well as by recognition of intermediate and final targets [148]. Additional interesting complex decisions regulated by guidance molecules are axonal fasciculation and de-fasciculation events.

All of the above described major axonal tracts, such as the TCA and the corpus callosum, require the existence of a former extending axon that pioneers the route followed by subsequently migrating cells [154, 227]. Though the preference of outgrowing axons to migrate on an existing pioneer axon was described almost 100 years ago [228], the cellular and molecular mechanisms underlying this event are far from completely understood. This phenomenon is known as axonal fasciculation and once again evidence indicates that axon-glia interactions are crucial. Though glia generate a repulsive signal at turning points, such as in the midline, growing axons seems to favour the migration on immature glia, which are typically abundant in proximity to emerging tracts [229].

Axon-axon homotypic interactions are instead critical to create axonal subpopulations within bundles required for proper targeting. Homotypic fasciculation has been recently highlighted as a key mechanism for the spatial segregation of olfactory sensory neurons [230]. In order to achieve such a complex organization, a plethora of guidance molecules are involved to push together and pull apart axons during timed fasciculation and de-fasciculation events [154]. One class of guidance molecules important during fasciculation is the CAMs family. Among the CAMs, Fasciclin II in insects and NCAMs in vertebrates, are major determinants of correct axonal fasciculation [231]. Contemporarily, the environment surrounding migrating axons provides either a favourable substrate, facilitating the invasion of axonal growth cones, or repulsive cues that promote continued fasciculation [158].

Slit2 facilitates axon fasciculation *via* an autocrine/juxtaparacrine mechanism in motor axons, which exhibit a tendency towards premature de-fasciculation in Slit2 or Robo1 and Robo2 knockout mice [215]. In addition to guidance molecules axonal fasciculation is also regulated by ion channels [232], calcium activity [233] and endocannabinoids [234, 235]. Moreover, very recent studies have shown that axonal outgrowth is further sensitive to mechanical properties of the surroundings *via* the mechanosensitive ion channel piezo1 [151], to optical stimuli [236], electrical stimuli [150] and modulated by hybrid innovative approaches [237-239].

Solving the riddle of axonal pathfinding therefore requires a broad vision, able to embrace 100 years of classic molecular neuroscience on cytoskeleton dynamics and combine it to a variety of guidance cues, signalling cascades and heterogeneous cell populations. This is not merely an exciting scenario, but rather an urgent necessity since an increasing number of neurological disorders are related to axonal outgrowth deficits [240, 241].

1.3 ENDOCANNABINOIDS AND BRAIN DEVELOPMENT

The idea of endogenous cannabinoids as a fundamental neuromodulatory system emerged more than 30 years ago. However, the therapeutic properties of *Cannabis Sativa* (麻) were described in the oldest Chinese pharmacopoeia, the Shennong Bencaojing (ca 200 BC), the first written gathering of oral traditions dating back to the Chinese sovereign Shennong, who was said to live around 2800 BC [242]. Despite being known for almost 5000 years, the therapeutic value of cannabis was not evaluated scientifically until the beginning of the 19th century by the Irish physician Sir William B. O'Shaughnessy during his work in Calcutta [243]. The recognition of endogenous cannabinoids was however delayed until the psychoactive components of cannabis were purified: cannabidiol (CBD), first characterized in 1940 by the Nobel laureate Lord Alan Todd in the UK [244] simultaneously with the American colleague Roger Adams [245]; and THC, first isolated in 1964 by Raphael Mechoulam and Yechiel Gaoni [246]. After THC identification, almost two decades of synthetic chemistry were necessary in order to identify the first THC-binding site. In 1990 [247] an "orphan" G-protein coupled receptor (GPCR) was identified as first cannabinoid receptor in the CNS, followed 3 years later by the cloning of a second peripheral receptor for cannabinoids [248]. The isolation of the first endogenous ligand shorty followed in 1992, with the characterization of arachidonoyl ethanolamine, or anandamide (AEA) [249].

1.3.1 Endocannabinoid signalling machinery

1.3.1.1 Endogenous cannabinoids

The endocannabinoid system includes eCBs, cannabinoid receptors and the enzymatic machinery responsible for the synthesis and degradation of eCBs. Endogenous cannabinoids are a family of small signalling lipids able to bind cannabinoid receptors with functional similarities to the main psychoactive component of cannabis, THC. The first eCBs to be synthesized and the most studied to date are AEA [249] and 2-AG [250]. The family of endogenous cannabimimetic and related lipid mediators potentially contains hundreds of bioactive molecules [251], emphasizing how our current knowledge on the endocannabinoid system might only be the tip of the iceberg.

2-AG, on which this chapter focuses, is the most abundant cannabinoid in the mammalian brain [252]. Structurally, 2-AG is an ester formed from the essential ω-6 polyunsaturated fatty acid arachidonic acid and glycerol [250]. Interestingly, the precursors of eCBs are therefore part of the cellular lipid membrane. This is a unique feature of eCBs compared to other classic neurotransmitters, which are synthesized ahead of need and stored in synaptic vesicles. eCBs instead can be released in the extracellular space on demand, liberated from the lipid membrane in only a few enzymatic steps, upon cellular depolarization [253-255] or GPCR activation [256]. How endocannabinoids cross cell membranes and move extracellularly remains unanswered, though it was recently proposed that active endocannabinoids are present in secreted microvesicles [257].

eCBs are synthesized downstream from arachidonic acid, which itself is derived from an essential fatty acid. This raises an appealing and still poorly studied consideration: because

long-chain polyunsaturated fatty acids (PUFAs) need to be introduced in our body by diet, that fluctuation in the composition of our diet could possibly modulate the availability of eCBs and related signalling molecules [258]. A further development of this concept could produce interesting considerations on ω-6 enriched western diets [259] and possibly explain the increasing number of studies that correlate fatty acid diet composition to behavioural outcomes [258, 260-263].

1.3.1.2 Endocannabinoid receptors

The functional effects of endo- and phyto-cannabinoids are principally mediated by CB_1Rs, primarily localized within the CNS, and by CB_2Rs, highly expressed in the peripheral systems as well as in the CNS in lower concentrations. Peroxisome proliferator activated receptors (PPARs) and transient receptor potential channels (TRP), as well as other receptors not detailed in this section, were also shown to mediate some actions of eCBs [264]. Both TRP channels, particularly TRPV1, and PPAR-α /PPAR-γ receptors are activated by AEA, with major effects on gene transcription [265, 266]. 2-AG and AEA have specific and unique affinities towards different endocannabinoid receptors. While 2-AG is a high-efficacy agonist of CB_1Rs and CB_2Rs, AEA is a low-efficacy agonist of both receptors. CB_1Rs and CB_2Rs, both GPCRs, once activated primarily couple to G_i and G_0 proteins, further activating intracellular cascades and inducing several cellular functions (for an extensive review on cannabinoid receptors see [267]). Cortex, basal ganglia, hippocampus and cerebellum regions display the highest expression of CB_1Rs [267], cumulatively considered the most abundant metabotropic receptor in the brain [268] . Subcellularly, CB_1Rs avoid the active zone, despite enrichment within presynaptic terminals [269]. While CB_2Rs are mainly involved in peripheral functions, they are sparsely expressed in the CNS where they are classically described as being restricted to microglia and vascular elements [270]. This traditional view has been challenged by emerging strong evidence that shows CB_2R mRNA in neuronal cells of the hippocampus [271] and in dopamine-expressing neurons in the ventral tegmental area (VTA) [272]. CB_2R-mediated modulation of cell type-specific plasticity was described in the hippocampus [273]. Moreover, CB_2R upregulation in neurons was observed under pathological conditions [274, 275].

1.3.1.3 Endocannabinoids synthesis and degradation

As cited above, AEA and 2-AG contain arachidonic acid backbone. However while 2-AG is synthesized from 2-arachidonoyl-containing phospholipids, most AEA is produced from N-arachidonoyl phospatidyl ethanol (NAPE) [276]. The enzymatic machinery involved in their metabolic pathways is therefore completely distinct. AEA is synthesized *via* multiple pathways which were shown to be brain region specific or related to certain physiological or pathological states [276]. Four main routes of AEA synthesis have been described, the first of which was by hydrolysis of NAPE *via* NAPE-phosholipase D (NAPE-PLD) [277]. Subsequent studies confirmed that NAPE-PLD ablation affects AEA levels and causes CB_1R redistribution [278]. Alternatively, AEA can be synthesized by cleavage of the phosphodiester bond of NAPE *via* NAPE-PLC and sequential dephosphorylation, a pathway mainly characterized in immune cells [279]. Finally two additional routes of AEA synthesis are i) dual hydrolysis of NAPE by the

α/β domain-containing hydrolase 4 (ABHD4) followed by an extra hydrolysis by glicerophosphodiester phosphodiesterase I (GDE-1) [280]; and ii) one hydrolysis step by ABHD4 followed by AEA liberation *via* lyso-NAPE-PLD [281]. Even if mechanistically described, the roles of the last three synthesis mechanisms in the CNS remain to be determined. There are two major routes of AEA degradation: *via* fatty acid amide hydrolase (FAAH), the major route [282]; and alternatively *via* AEA oxidation through cyclooxygenase-2 (COX-2) [283].

Most 2-AG is synthesized by hydrolysis of arachidonoyl-containing phosphatidyl inositol bis-phosphate *via* PLCβ, resulting in a diacylglycerol (DAG) that is sequentially hydrolysed by a diacylglycerol lipase (DAGL) [284]. This last step can be mediated either by DAGLα, seemingly responsible for most of the 2-AG synthesis in the adult CNS, or by DAGLβ that is able to cooperate with DAGLα in specific conditions [285]. 2-AG can be hydrolytically degraded primarily by MAGL, or alternatively by ABHD6 and by ABHD12, each localized within specific subcellular compartments [286]. MAGL is responsible for the majority of 2-AG degradation in the brain [286]; it is highly expressed in the CNS and mainly localized at the synaptic terminals [287]. In contrast, ABHD6 is expressed in dendrites and dendritic spines of pyramidal cells in the cortex [288]. Inhibition of either MAGL [289] or ABHD6 [288] can increase 2-AG signalling *via* CB_1Rs. Though the ABHD12 metabolic pathway has been molecularly characterized [290], its function *in vivo* remains to be established. Alternative pathways of 2-AG degradation are *via* COX-2 oxidation [291] or FAAH hydrolysis [292]. Historically, DAGLα and MAGL have been considered key enzymes in neuronal regulation of 2-AG, exhibiting synaptic segregation within pre- and post-synaptic compartments respectively [287].

In addition to its role in eCB signalling 2-AG also participates in a wide range of other cellular functions such as prostaglandin synthesis [293], which should be considered when manipulating 2-AG levels [276].

1.3.1.4 New insights from CB_1R pharmacology

Despite major efforts invested in the last 25 years delineating CB_1R modes of action, recent data reported in this section suggest that further investments might be necessary. Indeed, new advances in CB_1R pharmacology have revealed several natural and synthetic orthosteric agonists, such as CP-55,940, antagonists, including rimonabant, and additional endogenous cannabinoids [294]. Therapeutic application of rimonabant and other eCB ligands revealed significant side effects in humans [295]. This generated interest to develop novel neutral CB_1R antagonists, known as CB_1R blockers [296]. Complicating matters are additional findings demonstrating that eCBs also target non-cannabinoid receptors such as G protein-coupled receptor 55 (GPR55) [297], as well as ion channels including vanilloid, serotonergic and nicotinic receptors [294].

Novel therapeutic approaches for manipulating eCB signalling are focusing on putative allosteric binding sites and the development of both positive and negative CB_1R allosteric modulators which may be safer than full receptor agonists and antagonists [298]. Within this category indole derivatives, known as “ORG” compounds [299], and urea derivatives, such as PSNCBAM-1 [300], are being tested in animal studies. Natural allosteric modulators of CB_1Rs

have also been described, including lipoxinA4, the hemopressin, pepcan-12 and pregnenolone [301]. Moreover, historically known cannabinoids were recently re-discovered. CBD, a cannabis component historically considered a CB_1R antagonist, was recently reclassified to non-competitive negative allosteric modulator [302]. The increasingly accepted therapeutic potential of phytocannabinoid-based medicine demands further characterization of the actions of these compounds, including their potential allosteric functions.

CB_1R heteromerization with other proteins and receptors was recently shown to significantly affect *in vivo* eCB function, further complicating the picture. CB_1Rs have in fact been shown to generate heteromers with adenosine A_{2A} receptors in the striatum [303], with 5-HT2A serotonin receptors in memory related brain regions [304] and surprisingly with CB_2Rs [305]. Interestingly, cell membrane fluidity *via* lipid composition has been proposed as a critical regulator of CB_1R signalling in neurodegenerative disorders [306]. Even further complexity is introduced by observations that CB_1Rs can exist in a ligand-free constitutively active mode, which has been shown to tonically regulate GABA release in the hippocampus [307].

1.3.2 Endocannabinoid spatial and functional heterogeneity

1.3.2.1 The dogma: endocannabinoids are retrograde synaptic messengers

eCBs, particularly 2-AG, are historically categorized as retrograde messengers. This conclusion arose from observations describing that postsynaptic activity upregulates eCB production, that eCB machinery is synaptically compartmentalized and that CB_1R engagement inhibits synaptic transmission [268]. eCB-mediated synaptic plasticity includes depolarization-induced suppression of inhibition (DSI) and depolarization-induced suppression of excitation (DSE). DSI and DSE reflect transient suppression of GABA and glutamate release respectively following strong postsynaptic neuron activation and are reproduced in slice electrophysiology by a step depolarization or repeated action potential firing lasting a few seconds in postsynaptic neurons [308] [309]. eCB-mediated short-term plasticity *via* DSI and DSE were described first in the hippocampus [310, 311] and the cerebellum [309] in 2001, promptly followed by numerous attempts to characterize underlying molecular mechanisms [268]. Exogenous cannabinoid driven activation of these mechanisms were also assessed by numerous studies [312, 313].

Metabotropic-induced suppression of inhibition (MSI) and metabotropic-induced suppression of excitation (MSE), also known as synaptically evoked suppression of inhibition and excitation, were later described as ubiquitous forms of short-term plasticity [268]. MSI and MSE require by definition the engagement of a variety of G_q/11-linked GPCRs, including but not limited to metabotropic glutamate receptor 1/5 (mGluR1/mGluR5), M1/M3 muscarinic receptors, Cck_A, orexin A and $\alpha_1$adrenergic receptors [268]. The subsequent intracellular calcium dynamics activate calcium-sensitive PLCβ1 [314], resulting in diacylglycerol production and consequent release of 2-AG *via* DAGL. The newly synthesized 2-AG liberated in the postsynaptic cell then binds to and activates presynaptic CB_1Rs, which transiently suppress transmitter release [268].

eCBs can also drive long-lasting inhibition of synaptic transmission, known as eCB-mediated long-term depression (LTD). LTD is defined as homosynaptic if induced by eCBs generated locally within the stimulated cell, typically using persistent low frequency activation of glutamatergic synapses [315]. Heterosynaptic eCB-mediated LTD occurs in neighbouring synapses that are not activated during the conditioning stimulation. This form of metaplasticity has been described in the hippocampus, where excitatory drive from Schaffer collaterals can heterosynaptically decrease GABAergic input to principal cells [316]. Given the importance of LTD in sculpting cortical circuit development [317], disruptions in eCB-mediated LTD following cannabis abuse in adolescence may have severe consequences for network maturation and cognitive function.

Finally, neuronal excitability can be directly reduced by eCBs through slow self-inhibition (SSI) [318]. With intense stimulations, SSI induces 2-AG synthesis, somatic CB_1R activation with consequent recruitment of inwardly rectifying potassium channels [319]. Cell-autonomous SSI has been observed in low threshold-spiking cortical interneurons [318] cerebellar basket cells [320] and cortical excitatory cells [321].

1.3.2.2 Foci for action: endocannabinoids at $Cck^+CB_1R^+$ interneurons

CB_1R expression in the CNS is particularly high in the cortex, hippocampus, basal ganglia, and cerebellum. CB_1R preferential expression within cortical and hippocampal interneurons was established shortly after the development of a reliable CB_1R antibody [322] with two independent groups showing that CB_1R expression localized almost exclusively to Cck-containing GABAergic interneurons [323, 324]. Electron microscopy highlighted CB_1R^+ axon terminals of Cck-containing basket cells innervating the soma and proximal dendrites of pyramidal neurons [324]. These findings firmly established Cck presynaptic elements as a major substrate underlying eCB-mediated modulation of hippocampal network function. Subsequently, numerous studies aimed to dissect eCB-mediated synaptic modulation specifically in Cck interneurons in the hippocampus [325-329], amygdala [330, 331], midbrain [332] and hypothalamus [333].

Chole-cysto-kinin (from Greek, literally “bile-sack-move”) was identified in 1975 as a molecule of the gastrointestinal tract important for gastric motility [334]. Subsequently, Cck was identified as one of the most abundant neuropeptides in the brain, localizing in a cell-type specific manner [335, 336]. Cck is derived from its precursor molecule preprocholecystokinin [337], *via* extensive tissue-specific post-translational processing, typically yielding the predominant form of Cck in the brain: an 8-amino acid C-terminal fragment (Cck-8s) [338]. Cell-type specific Cck expression has been extensively studied in the hippocampus where expression is enriched in subsets of GABAergic cells typically referred to as “Cck interneurons” [339]. In the hippocampus, one subset of Cck interneurons provides perisomatic input to principal cells, thus regulating pyramidal neuron output and synchrony [340]. Because of the shape of their axons, these Cck interneurons fall into the category of basket cells, innervating selectively the soma and proximal dendrites of postsynaptic targets [340].

Another family of basket cells, PV-expressing interneurons, has a dominant role in pacing and synchronizing network oscillations [341]. In contrast, Cck basket cells are considered to act as "fine tuning" modulatory cells, able to receive and integrate inputs from several subcortical regions [341]. Cck protein has also been identified in pyramidal excitatory cells in the neocortex and the hippocampus, able to modulate glutamatergic excitatory system, possibly facilitating glutamate release (for more details on Cck functions in excitatory neurons see [342, 343]).

While convenient as an interneuron subtype marker, the role of Cck neuropeptide is not yet completely understood. Studies on Cck peptide functional roles highlighted two principal mechanisms of action: *via* PV interneurons and within Cck basket cells themselves [344]. Cck-8s acute application on hippocampal slices was shown to increase pyramidal cell spontaneous postsynaptic inhibitory event (sIPSCs) frequency [345, 346] mediated by a selective robust activation of PV-expressing basket cells *via* Cck2 receptors [345]. When the Cck peptide was instead applied during paired recordings between Cck basket cells and postsynaptic pyramidal cells, inhibition of unitary IPSCs was observed [345]. The proposed mechanism behind this phenomenon involves an indirect functional interaction between Cck peptide and eCBs. Specifically, Cck receptor activation in postsynaptic pyramidal cells couples through $G_{q/11}$-protein signalling to drive 2-AG synthesis and release, which then binds to presynaptic CB_1Rs within Cck basket interneurons [345]. Once activated, presynaptic CB_1Rs suppress GABA release by inhibiting N-type calcium channels through G_i-protein coupled signalling [324]. Cck peptide application therefore promotes an imbalance in pyramidal cell perisomatic inhibition by coincidentally increasing and decreasing output from PV and Cck basket cells respectively [327, 347]. However, similar modulation of inhibitory tone by endogenous Cck has yet to be demonstrated. CB_1R engagement was shown to inhibit the release of numerous peptides including Cck [348]. Using combinatorial pharmacological modulation of Cck and CB_1Rs by selective antagonists, a recent study suggests that CB_1R-Cck peptide interactions participate in conditioned fear extinction, anxiety and stress [349].

Interestingly, $Cck^+CB_1R^+$ interneurons were identified as an early population in brain development originating from the CGE at embryonic day E12.5 [350]. Morozov and colleagues highlighted an early commitment to the $Cck^+CB_1R^+$ phenotype and the following three-phasic migratory pathway: from VZ of the ventral telencephalon to the MZ of the dorsal neocortex, into the hippocampus. $Cck^+CB_1R^+$ cells migrate tangentially to the hippocampal primordium, where they arrive between E13.5 and E15.5 [350]. Because of their early CB_1R expression, foetal eCB imbalances could dramatically impair the migration and differentiation of these Cck expressing cells, potentially precipitating circuit wide deficits in network activity.

1.3.2.3 Not that simple: heterogeneous distribution of CB_1R

Although CB_1Rs are primarily localized presynaptically in Cck basket cells and the majority of the literature focuses on CB_1R-mediated regulation of neurotransmitter release, recent evidence points to additional cellular substrates that can mediate eCB functions. For example, somatodendritic CB_1Rs were shown to control *via* self-inhibition specific

postsynaptic signalling cascades in the cortex [318, 319] and mediate eCB-induced cognitive impairments [321]. Indeed, CB_1R expression is no longer considered exclusive to Cck basket cells, as these receptors have now been well characterized in glutamatergic [351] and serotoninergic neurons [352]. Dopamine [353] and acetylcholine [354] release are also regulated by eCBs. CB_1R expression within these varied cell types does not necessarily reflect an equal distribution, as evidenced by differential CB_1R expression in brain regions and within cell types [267, 340]. Such a regulated and heterogeneous distribution likely contributes to the variety of functional effects of eCBs [276] and may partially underlie the bimodal effect of cannabis [355].

Further complicating the picture are recent findings suggesting that functional CB_1Rs are not restricted to the plasma membrane but also expressed intracellularly, as exemplified in mitochondria where CB_1Rs regulate neuronal energy metabolism (for a recent review on neuronal activity and mitochondrial CB_1R see [356]). CB_1Rs expressed in the outer membrane of mitochondria ($mtCB_1R$) [357] are able to engage a signalling cascade *via* cyclic-AMP, PKA and complex I triggering neuronal respiration decrease and inhibition of memory formation [358]. Though the mechanistic details of $mtCB_1Rs$ were recently delineated [358], functional implications of $mtCB_1R$ signalling require further investigation.

Peripheral CB_1R expression within a variety of tissues has been extensively assessed [359-363]. Only recently however, functional interactions between brain-controlled behaviours and peripheral eCB-mediated processes have been discussed. CB_1R-mediated sympathetic activation was indeed shown to mediate hypophagia and anxiety like behaviours [364]. Furthermore, CNS-periphery CB_1R-mediated signalling was shown to modulate stress hormone release from the adrenal medulla [365] and gut microbiota metabolism in obesity [366, 367].

1.3.2.4 Glia-neuron interplay regulates endocannabinoid actions

Cannabinoid receptor expression in glial cells challenged the initial neuro-centric view of the eCB system. Moreover, though initial evidence suggested that CB_1Rs selectivity localized to neurons while CB_2Rs are restricted to glia, recent findings indicate a more heterogeneous distribution within cell types [368]. CB_1R expression in astrocytes was shown to have a principal role in modulating neuron-glia interactions, able to regulate LTD and learning and memory [368, 369]. Mechanistically, this was found to be related to MAGL-mediated 2-AG metabolism [369]. Hence, CB_1R modulation of synaptic plasticity has to be reconsidered, adding astrocyte-mediate signalling to the already described pre- and postsynaptic mechanisms. CB_1R signalling has also recently been demonstrated in microglial cells [370] and in oligodendrocyte progenitors whose survival is enhanced by eCBs *via* Akt signalling [371]. CB_1Rs, CB_2Rs, DAGLα/β and MAGL are all expressed in developing oligodendrocytes and appear to be developmentally regulated [372], highlighting roles for 2-AG metabolism and signalling in oligodendrocyte progenitor development.

1.3.3 Endocannabinoids in the developing neurite

In addition to serving as retrograde signals at adult synapses, eCBs also play critical roles in neurodevelopment. eCBs contribute to cell proliferation [373], migration [374], axonal outgrowth [375] and synaptogenesis [376]. The molecular mechanisms underlying these diverse roles of eCBs in the developing brain are area of intense research.

1.3.3.1 Endocannabinoid signalling machinery in the foetal brain

eCB machinery varies substantially throughout brain development and considerably differs from its adult counterpart. Endogenous 2-AG levels in rodents are relatively stable from the prenatal stage throughout adolescence and adulthood (2-8 nmol/g tissue), with a noticeable peak at birth [377, 378]. AEA levels are high at fertilization, followed by a strong intrauterine downregulation, important for embryonic implantation [379]. AEA is then detectable again from mid-gestation onwards, steadily increasing from the perinatal period (3-6 pmol/g) until adulthood [377]. Understanding the functional implications of these changes in AEA requires consideration of the varied eCB mechanisms of action within developing neurons and glia. In particular, immature developing neurons exhibit a unique cell autonomous (autocrine) eCB signalling mechanism, in contrast to the above-described paracrine eCB synaptic signalling.

CB_1R, CB_2R, DAGLα and DAGLβ are all co-expressed within neuronal progenitors in the SV zone, consistent with an autocrine eCB signalling mechanism regulating asymmetric cell division [380, 381]. Furthermore, the eCB degrading enzymes MAGL and FAAH are highly expressed in radial glia [375] and progenitor cells [373]. Once progenitor cells differentiate to neuronal cells a CB_2R to CB_1R switch occurs, coincident with downregulation of DAGLα and DAGLβ, thus promoting cellular sensing of 2-AG concentration gradients to stimulate cell migration [380] (Figure 7).

In mice, CB_1R mRNA has indeed been detected in pyramidal cells of the cortical plate form E11.5, with a peak at E14.5 [374]. Moreover, at E12.5, cells in the subpial area of the GE, putative early interneurons, intensely label for CB_1Rs [350]. These patterns of expression were also confirmed in human studies, which detected CB_1Rs in the cortical plate at early stages of development (9–10 pcw) within radially oriented putative pyramidal cells, and in immature interneurons at early stages of cortical development (9–17 pcw) [382]. It is interesting to note that the subcellular localization of eCB receptors was cell type specific [376]. Moreover outgrowing axonal projections both in human and rodent transiently express CB_1Rs that are downregulated after synaptogenesis [378].

Changes in eCB enzymatic machinery expression profiles are also instructive for neuronal development, driving a switch from autocrine to paracrine signalling [381]. DAGLα and DAGLβ are co-expressed with CB_1Rs at mid-gestation in the axonal shaft and growth cones of corticofugal migratory neurons [285]. MAGL expression was detected within central and peripheral axons as early as E12.5 [375]. Since CB_1Rs and DAGLα coexist along the axonal shaft, 2-AG inactivation and sequential availability is determined by MAGL subcellular position. Indeed, MAGL is generally excluded from the growth cone during migration, when it accumulates in the axon stem, generating a decreasing gradient of 2-AG hydrolysis towards

the growth cone [375]. Once migratory neurons reach their target structures, eCB signalling further instructs the development of synaptic contacts. During synaptogenesis MAGL redistributes in the growth cone contributing to stop axonal growth by eliminating 2-AG signalling at the tips. Simultaneously, DAGLα localization switches from axonal to somatodendritic and postsynaptic, generating focal loci for 2-AG production, to signal through CB_1R^+ presynaptic terminals of GABAergic cells [285]. The adult synapse is therefore established with the characteristic presynaptic expression of CB_1Rs and MAGL; while DAGLα localises at its postsynaptic counterpart [381]. This enzymatic machinery redistribution orchestrates a functional switch from autocrine to paracrine eCB signalling [381].

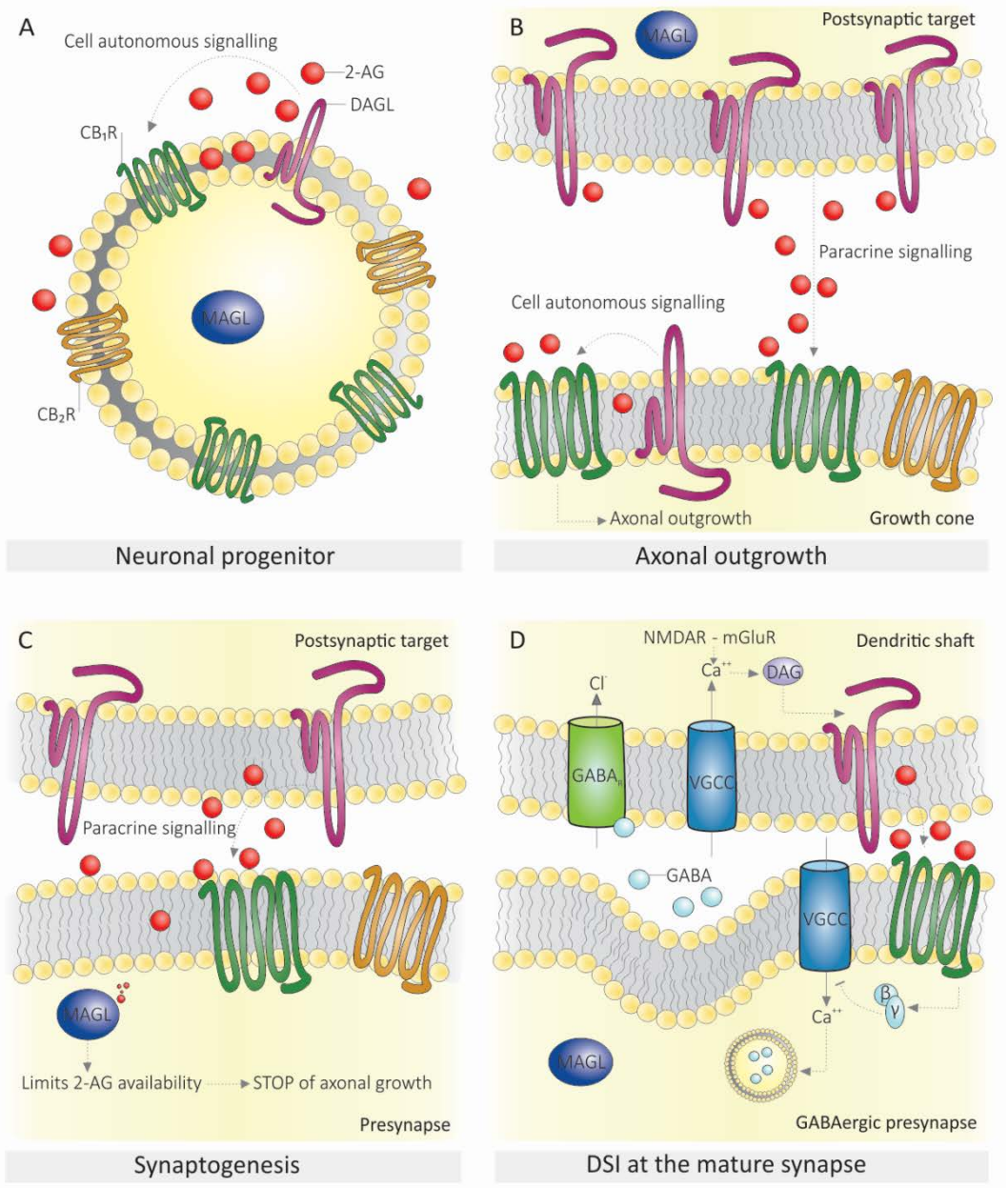

Figure 7. Endocannabinoid signalling shifts form autocrine to paracrine along neuronal development. 2-AG receptors and enzymatic machinery distribution differs substantially throughout different stages of brain development and synaptogenesis. **(A)** DAGL (magenta), MAGL (blue), CB_1R (green receptor) CB_2R (orange receptor) are co-expressed in neuronal progenitors, promoting cell

autonomous 2-AG (red circles) signalling and asymmetric cell division. **(B)** Once neurons exit the proliferation stage and start migrating, CB_1Rs mainly characterize the developing neurite. Cell autonomous 2-AG signalling is still present at the beginning of neurite outgrowth, where CB_1Rs mediate intracellular signalling leading to cytoskeleton modification and axonal outgrowth (for a comprehensive review on 2-AG-induced signalling *via* CB_1R see [383]). MAGL is generally excluded from the growth cone. Accumulating along the axonal stem (not shown), MAGL generates a decreasing gradient of 2-AG hydrolysis towards the growth cone, essential for correct growth cone extension [375]. Along cell development DALG is gradually downregulated in the growth cone, favouring a paracrine signalling and migration along the 2-AG gradient generated by the target cells, expressing high levels of DAGLα and MAGL. **(C)** Once the growth cone reaches the postsynaptic target proximity, the endocannabinoid machinery further rearranges to mediate the correct pre- and postsynaptic compartments apposition. During synaptogenesis MAGL redistributes within the presynapse, limiting 2-AG availability and likely contributing to generate the stop signal for axonal outgrowth. At this stage DAGL is excluded from the presynaptic compartment and redistributes to its final location within the soma and the dendrites. Therefore at this stage 2-AG is provided exclusively by the postsynaptic target cell, that *via* paracrine signalling promotes synaptogenesis. **(D)** eCB signalling within the mature synapse is quite complex and varies depending on the type of cells engaged, mediating plasticity *via* multiple mechanisms. Here, depolarization-induced suppression of inhibition (DSI) is illustrated, one of the most studied eCB-mediated retrograde signalling events. Postsynaptic depolarization by excitatory inputs causes an increase of intracellular Ca^{++} by activation of voltage gated calcium channel (blue) *via* NMDA receptors and mGLU receptors. This excitation-induced cascade leads to 2-AG production, *via* DAGL in the postsynaptic compartment. Released 2-AG binds to $G_{i/o}$-coupled CB_1Rs expressed on the GABAergic presynapse and inhibits Ca^{++}-dependent GABA release, ultimate yielding DSI.

1.3.3.2 The role of endocannabinoids in neurite outgrowth

Numerous genetic and pharmacological approaches have confirmed a fundamental role for eCBs in axonal outgrowth, cell migration and target recruitment (Table 1). Exogenous cannabinoids applied *in vitro* can trigger CB_1R internalization and downregulation from sensing structures such as filopodia, resulting in a collapsed growth cone and overall chemorepulsion [375]. Genetic ablation or pharmacological inhibition of CB_1Rs leads to fasciculation defects of the major axonal tracts within the cortex [374]. Moreover, CB_1R interneuron-specific downregulation resulted in a redistribution of inhibitory synaptic puncta in the adult cortex, representing a long-lasting failure in targeting cortical structures [376].

Intracellular mechanisms involved in cannabinoid–induced axonal outgrowth were recently addressed, highlighting an indirect cannabinoid-cytoskeleton interaction [384, 385]. Both endogenous and exogenous cannabinoids were able to trigger CB_1R coupling with $G_{12/13}$ proteins, leading to actomyosin-mediated cytoskeleton contraction *via* non-muscle myosin (NMII) through a Rho-GTPase and Rho-associated kinase (ROCK) [384]. This proposed intracellular mechanism results in rapid growth cone and neurite remodelling, increased cytoskeleton stability and an overall impairment of somatodendritic morphology [384].

Using a proteomics approach, a second study proposed a similar mechanism in mouse cortex [385]. CB_1Rs were shown to interact and regulate the activity of multiple members of the WAVE1 complex and the Rho GTPase Rac1 [385]. In this study, growth cone retraction was

induced *via* WAVE1 upon CB_1R engagement, leading to growth cone collapse in developing neurons and spine remodelling in mature neurons [385]. Interestingly, a recent study suggests that the contribution of eCBs to axonal outgrowth is not solely mediated by CB_1Rs, but also by CB_2Rs [386]. CB_2Rs were localized to the retino-thalamic pathway, specifically within axonal growth cones [386].

Genetic and pharmacological modulation of CB_2Rs induced growth cone reorganization *via* a cAMP/PKA pathway and impaired retino-thalamic pathway development [386]. DAGLα co-expression with CB_1Rs in the growth cone during axonal growth is required to promote axonal outgrowth, ensuring sufficient 2-AG availability [376]. Indeed genetic deletion of DAGLα impairs cortical projecting bundles [387].

An example of the importance of eCB machinery localization is the developing thalamocortical bundle, characterized by an eCB-mediated "handshake" between corticothalamic and thalamocortical fibres [234, 375]. Thalamocortical axons lack CB_1Rs and express MAGL, while corticofugal CB_1R^+ axonal tips lack MAGL. Corticofugal axons also express DAGLα and use the produced 2-AG both in a paracrine manner, to control fasciculation, and as autocrine signalling at the tips to promote axonal extension. The hydrolytic activity of $MAGL^+$ within thalamocortical fibres limits 2-AG availability generating an eCB gradient responsible for the "handshake" mechanism *via* favourable migratory corridors [375].

Model	Observed phenotype
$CB_1R^{-/-}$	The first description of $CB_1R^{-/-}$ mice detailed an increased mortality upon CB_1R deletion, but no major developmental phenotype [388]. This idea was rapidly modified in follow up studies using conditional knockout mice, with migratory phenotypes and mistargeting of $CB_1R^{-/-}$ interneurons [376], progenitor proliferation impairments [376, 389] and axonal fasciculation defects in $CB_1R^{-/-}$pyramidal cells [390].
$CB_2R^{-/-}$	$CB_2R^{-/-}$ mice showed abnormal eye-specific segregation of retinal projections in the dorsal lateral geniculate nucleus, demonstrating CB_2R contributions to neuronal development [386].
$DAGL\alpha^{-/-}$	$DAGL\alpha^{-/-}$ mice revealed altered synaptic distributions in the hippocampus and axonal growth cone impairments [391].
$GPR55^{-/-}$	GPR55 genetic knockdown did not highlight any developmental anatomical axonal phenotype, rather inducing late-onset behavioural phenotypes, including memory impairment and depressive symptoms with major axonal phenotypes [392]. However a recent study assessed GPR55 modulation of growth rate of retinal projections using both $GPR55^{-/-}$, lysophosphatidylinositol (LPI) and O-1602 administrations (GPR55 agonists) [393].
AM251	Results achieved by pharmacological *in vivo* modulation partially recapitulate receptor knockdown models. Perinatal AM251 (CB_1R antagonist) injection was shown to affect critical period plasticity of the whisker map by mistargeting somatosensory axons of the barrel cortex [394].
SR414716	SR414716 (CB_1R antagonist) intraventricular prenatal injection in mouse embryos negatively affects progenitor migration from the subventricular zone and recapitulates corticofugal phenotypes described in $CB_1R^{-/-}$ mice [374].
WIN55,2122	Using the opposite approach, WIN55,2122 (CB_1R and CB_2R agonist) injection during pregnancy altered the migration of GABAergic interneurons and pyramidal cells as well as increased the number of migrating Cajal-Retzius cells along the MZ [395].
JZL184	MAGL inhibition consequences for development were addressed by JZL184 exposure (MAGL inhibitor). JZL184 acted as a functional antagonist causing an increase in available 2-AG and long-lasting effects on eCB receptor expression, with consequent axonal growth defects and impairments in cortical network formation [391].
URB597	Similarly to GPR55 genetic knockdown, URB597 treatment produced no major axonal phenotype, but did lead to long-lasting behavioural impairments [396].

Table 1. Untying endocannabinoids cobweb: a schematic summary of genetic and modulation studies. As described above, endocannabinoids are major players in axonal outgrowth. This table reports a schematic summary of genetic and pharmacological evidences supporting the importance of eCB and eCB machinery components in neuronal development. Modified from *Maccarrone et al* [381].

1.3.3.3 Endocannabinoids: key mediators of maternal programming

The central role of eCBs in critical events during brain development is extensively highlighted by the findings listed above. However, whether maternal eCB imbalances during pregnancy are sufficient to trigger developmental deficits that precipitate neurological disorders remains poorly investigated?

If so, blood-born circulating maternal eCBs could contribute to tonic embryonic eCB levels, while acute synthesis in specific foci of the developing brain would contribute to phasic localized eCB signalling. The foetal placenta represents not only an environmental barrier but also a key regulator of maternal-foetal interactions, as shown for nutrient transport, endocrine function and immune tolerance [397]. CB_1R, CB_2R and FAAH expression were recently assessed immunohistochemically and by mRNA levels in the human placenta and gestational membranes. Strong CB_1R expression was found in amniotic epithelium, reticular cells and moderate expression was observed in chorionic cytotrophoblasts [398]. FAAH expression was highlighted in amniotic epithelial cells and trophoblasts [398] and exhibited temporal regulation throughout pregnancy, with peak mRNA levels observed at 11 pcw before declining [399]. CB_2Rs were mainly localized within placental macrophages [399].

Clinical studies have confirmed strong correlations between decreased maternal circulating FAAH and early pregnancy success rate [400]. A separate study reported that significantly lower circulating levels of FAAH correlate with *in vitro* fertilization-embryo transfer failure [401]. Experimental models confirmed these finding, suggesting that low circulating FAAH activity is responsible for increased intrauterine AEA and consequent uterine refractoriness to blastocyst implantation [402, 403]. The above observations provide strong clues that maternal eCB machinery influences embryonic eCB availability.

Active transportation *via* placenta has been assessed for water, electrolytes, glucose, cholesterol and PUFAs. Placental PUFAs transportation is regulated by several plasma membrane-located transport proteins that in turn are regulated by fatty acid-activated transcription factors [404]. It is therefore possible that metabolic derivatives of maternal long-chain fatty acids are transported to the intrauterine environment, where the foetus can use them as metabolic precursors of eCBs. Indeed fatty acid composition of the maternal diet during the first and the second half of gestation was shown to determine fatty acid composition of mothers' milk and plasma, as well as fatty acid levels in offspring plasma [405]. Moreover, maternal dietary fat composition was repeatedly shown to alter fatty acid composition of cell membranes in the offspring brain [406-409]. Human ω-3 deficiency during gestation and lactation was shown to impair visual and cognitive functions in the offspring [410, 411]. Along the same line, ω-3 deficient diets reduce dopaminergic and serotonergic neurotransmission in rodent offspring [412, 413]. Moreover, maternal ω-6/ω-3 ratios directly affect 2-AG levels in offspring hippocampus and hypothalamus altering eCB-mediated inhibition of stress responses [414].

Interestingly, eCBs in pregnancy may interact with maternal leptin hormone signalling. In adults leptin was shown to reduce appetite by inhibiting orexigenic neuropeptide [415] in the hypothalamus [416]. Circulating leptin was found to be increased in pregnant mothers [417]

and leptin receptors are expressed in mice embryos as early as E10.5 [418]. Leptin signalling is downregulated in the hypothalamus of offspring born to obese mothers due to decrease leptin receptor expression leading to compromised hypothalamic network development [419]. A role for eCBs in this story was introduced when leptin and leptin receptor in the hypothalamus were shown to regulate 2-AG and AEA levels, providing the first mechanistic connection between eCBs and leptin [416]. Potential leptin-induced eCB imbalances in the foetal brain due to maternal obesity remain an unexplored possibility, but would be consistent with observations that trans-placental leptin passage peaks coincident with synaptogenesis in rodents [420].

1.3.4 Endocannabinoids and neurodevelopmental disorders

Both preclinical and clinical studies strongly support eCB involvement in several neurodevelopmental disorders [421-423]. Though the aetiology and symptomatic profile of such diseases are not detailed here, this section briefly highlights several described alterations of the eCB system within anxiety, mood disorders, schizophrenia and autism (for comprehensive recent reviews on the above illnesses see [424-427] respectively). Because of the neurodevelopmental nature of these disorders, an early intervention *via* eCB modulation might be beneficial, supporting the recently proposed preventive approaches for psychiatric and cognitive disorders [98].

Common limitations of the clinical studies reported below are due to the nature of psychiatric disorders, which might explain complex and even contradictory results in different studies due to chronic *versus* absent medication, illicit substances use, post-mortem experimental methodologies and poor living conditions frequently experienced by these patients. Recent development of novel imaging techniques such as quantitative positron emission topography (PET) combined with the CB_1R selective radioligand MK-9470, might partially overcome typical hurdles associated with post-mortem studies [428]. Moreover, a major difficulty in studying neurodevelopmental disorders is the typically delayed symptomatic stage. The vast majority of available literature on psychiatric diseases focuses on patients monitored after exacerbation of the disease. However, even if extremely complex to collect, it would be interesting to accumulate neurobiological data *prior* to symptom onset, during childhood and prenatal development of high-risk subjects.

1.3.4.1 Anxiety

eCB involvement in anxiety disorders is complex and only partially understood. Preclinical findings highlight both anxiogenic and anxiolytic effects upon cannabinoid agonist exposure [423]. Summarizing recent literature, cannabinoid agonists tend to reduce anxiety-like behaviours in low doses and increase them with high doses [423]. Local prefrontal cortex (PFC) increases in AEA signalling reduce anxiety with low doses of methanandamide (AEA analogue, metabolically stable) [429] and URB597 (FAAH selective inhibitor) [430]. High doses of methanandamide and URB597 mediate instead anxiogenic-like behaviour, an effect recapitulated by lentivirus-mediated FAAH over-expression in the PFC [423]. Anxiety-related effects upon 2-AG metabolism are less-well understood. However, recent data suggests

effects similar to AEA based upon JZL184 administration [431]. The most exciting results emerged therefore from modulation of eCBs locally within the PFC. However, eCB regulation both in the ventral hippocampus [432] and in the dorsolateral periaqueductal gray [433] has been shown to control anxiety-like behaviours.

1.3.4.2 Mood disorders

Rimonabant (SR141716, CB_1R inverse agonist) was introduced as an anorexic drug, and then quickly withdrawn due to side effects including the induction of depressive episodes and mood variation [434]. Therefore, if recreational cannabis is widely used for its euphoric/relaxing effect, CB_1R block *via* rimonabant induced instead depressive episodes. Cortical CB_1R expression alterations post-mortem have been identified in mood disorder patients [435], however the data are inconsistent across studies [423]. eCB levels and eCB machinery were found to be altered in a region-specific manner, with AEA and 2-AG increases in PFC [436] and higher FAAH activity in the ventral striatum [437] of depressed alcoholics compared to alcoholics without depression. Accordingly, pharmacological inhibition of FAAH enzyme reduced depressive-like behaviours in animal models [438]. Peripheral serum levels of AEA and 2-AG were significantly reduced in depressed patients, with an interesting negative correlation between 2-AG concentrations and length of the depressive episodes [439]. These findings could lead towards a future of personalized medical treatment for patients that present altered eCBs measured by blood samples [422]. Genetic screening in mood disorder patients revealed positive correlations between disease susceptibility and polymorphisms in CB_1R and FAAH encoding genes [423]. Studies on eCB imbalances in bipolar patients remain rare and are so far not conclusive [422]. Interestingly, major depressive disorder patients display alterations in peripheral lipid availability [440], including decreases in ω-3 fatty acid derivatives. Consistently, a recent cohort exhibited an inverse relationship between ω3 fatty acid intake and depression, confirmed by lower depression scores associated with an ω3 rich Mediterranean diet [441].

1.3.4.3 Schizophrenia

Among the neurodevelopmental disorders discussed herein, schizophrenia is the pathology most closely linked with dysfunction of the eCB system [423]. Despite numerous early observations suggesting that cannabis use worsens schizophrenia or induces psychotic symptoms [442], there is no direct evidence that cannabis is either sufficient or necessary to induce the disease [443]. Interesting findings on disease aetiology came instead from studies of eCB levels in patients and animal models. Cerebrospinal fluid (CSF) levels of AEA were significantly elevated in a cohort of antipsychotic-naive first episode schizophrenia subjects [444], as well as in a cohort of subjects with prodromal psychosis [445] and an additional cohort of subjects with acute symptoms off-medications [445]. However, the implications of increased peripheral AEA for the CNS remain to be addressed. Moreover, altered eCB levels were reported in blood samples [446] and post-mortem brain tissues [423] of schizophrenic patients.

Dysfunctions of dorsolateral PFC layer 3 local networks are chiefly involved in schizophrenia [443]. Indeed, an excitatory-inhibitory imbalance within layer 3 PFC has been confirmed by numerous observations, including a significant decrease of PV-basket cell inputs on layer 3 excitatory neurons in PFC of schizophrenic patients [447]. Moreover, the PFC of schizophrenic patients is characterized by lower PV mRNA and protein expression accompanied by an overall deficit in the GABA synthesizing enzyme glutamic acid decarboxylase 67 (GAD67) at mRNA and protein levels [443]. In addition Cck-basket cells have been recently proposed as active players in schizophrenia, based on observed reductions in Cck mRNA and protein levels in the PFCs of schizophrenic patients [448]. While DAGLα, DAGLβ, MAGL and FAAH levels remain unchanged in schizophrenic patients, ABHD6 mRNA levels were elevated in the PFC [443]. Interestingly, ABHD6 co-localize with DAGLα in dendritic spines of PFC excitatory neurons [288]. Thus, it was proposed that increased ABHD6 activity in schizophrenic patients could promote higher 2-AG metabolism at the foci of action, with a consequent reduction in 2-AG mediated activation of CB_1Rs in Cck-basket cells [443]. This may be consistent with human studies reporting lower CB_1R mRNA [423] but higher CB_1R binding by radiolabelled ligands [449], possibly reflecting altered trafficking of CB_1Rs upon decreased 2-AG availability (for a comprehensive review on eCBs-mediated inhibition impairment in schizophrenia see [443]). These observations suggest that pharmacological modulation of eCBs in the PFC could represent a valid novel opportunity for schizophrenia treatment. Indeed exciting results indicate that CBD is able to reduce negative symptoms in schizophrenic patients, possibly due to its ability to regulate AEA metabolism [423].

1.3.4.4 Autism spectrum disorders

eCB signalling in relation to autism spectrum disorders (ASD) has been recently reviewed [450]. Observed eCB-mediated regulation of emotional responses [451], context reactivity [452] and social interactions [453] in healthy subjects have suggested a possible involvement in ASD. Evaluation of eCB machinery in an ASD genetic model revealed dysfunctional 2-AG metabolism upon fragile X mental retardation (*fmr1*) gene knockout, together with decreased DAGL and MAGL activity [454]. Indeed, stimulating 2-AG metabolism restored synaptic activity in ASD models *via* type I metabotropic glutamate receptor activation [455]. Rat prenatal exposure to valproic acid (VPA, mood stabilizer) provides an alternative experimental model for ASD [456] and recapitulates the above described DAGL and MAGL enzymatic imbalances [457]. AEA-mediated autistic-like behaviours were mechanistically examined recently, demonstrating altered CB_1R phosphorylation in multiple brain regions, together with long lasting effects on FAAH and NAPE-PLD levels from infancy to adulthood in the VPA-ASD model [457]. Targeting AEA inhibition was therefore sufficient to rescue behavioural deficits of VPA-exposed rats [457]. Preliminary data also suggest CB_2R, GPR55 and PPAR involvement in ASD [450]. Moreover, plasma levels of unsaturated fatty acids are decreased in autistic patients [450], suggesting that supplement-based therapy may improve symptomology.

1.3.5 Prenatal cannabis exposure

1.3.5.1 Maternal cannabis prevalence: a socio-geo-political perspective

Reports on drug abuse highlight a noticeable State-to-State variability in cannabis consumption. About 147 million people or 2.5% of the world population, use cannabis at least annually, compared with 0.2% that consume cocaine and opiates [458]. A 2016 cannabis usage prevalence map of Europe depicted a heterogeneous scenario [459]. Cannabis prevalence was reported for all adults (15-64), young adults (15-34 years) and school age respectively, for UK (7%, 12%, 25%), France (11%, 22%, 39%), Spain (9%, 17%, NA), Germany (5%, 11%, 19%), Italy (9%, 19%, 21%), Austria (4%, 7%, NA), Netherland (8%, 16%, 27%), Hungary (2%, 6%, 19%) and Czech Republic (11%, 24%, 42%) [459]. European cannabis usage prevalence has remained stable within the last few years in certain countries (eastern and south-eastern Europe), decreased in others (Western and central Europe) and increased in a few (Bulgaria, Estonia, Finland and Sweden) [459]. The 2016 National Survey of Drug Use in the USA reported cannabis usage ranging from 10.4% (26 years-older) to 32.2% (18-26 years), with a 1% increase from 2013 to 2015 [460].

Global prevalence fluctuations are certainly affected by the mutable social and legal status of recreational cannabis consumption. Cannabis has historically been classified as an illegal narcotic drug in Europe from 1961 (United Nations single convention on narcotic drugs) and illegal under federal law in the United States, where it is classified as a Schedule I substance under the Controlled Substances Act of 1970. However, national autonomy on this matter has resulted in a very heterogeneous "legal map" for recreational cannabis. Legality of cannabis use is quite heterogeneous even within the US, with a recent update in November 2016, when 8 sates fully legalized both recreational and medicinal marijuana (Alaska, California, Colorado, Maine, Massachusetts, Nevada, Oregon and Washington). The remaining states report decriminalized policy and often legalized medical use. The European portrait is also complex with variable legal positions for recreational cannabis, including: 1) tolerance for possession of small amounts intended for personal consumption (Belgium, Germany, Greece and Austria); 2) small amount consumption allowed only in "Coffee shops" (Netherlands) or "Cannabis Social clubs" (Spain); 3) administrative sanctions (Denmark, Czech Republic, Estonia, Ireland, Italy, Latvia, Luxemburg, Portugal, Slovenia, Croatia and Norway); and 4) penal sanctions (Cyprus, Hungary, Poland, Slovak Republic and UK) [459]. Medical cannabis approval is quickly spreading in Europe as well including cannabis plant smoking, whole plant extracts (Bedrocan or Nabiximols, trade name Sativex), and THC synthetic analogues (Nabilone and Dronabinol, marketed as Marinol and Cesamet) approved as anti-nausea, antiemetic, antispasmodic, anti-inflammatory, anti-epileptic and analgesics [459].

While global cannabis use, as for most of illegal drugs, remains more popular in males, alarming results are reported from cannabis consumption in females during pregnancy. A recent study reported a meta-analysis of cannabis consumption from the female cohort aged 18 to 44 years of the annual National Survey on Drug Use and Health (NSDUH, 2002 to 2014). Among all pregnant women prevalence of past-month marijuana use increased 62% from 2002 through 2014, rising from 2.37% in 2002 to 3.85% in 2014. Prevalence of past-month

marijuana use in 2014 was significantly higher in pregnant younger women (reaching 7.47% among those aged 18 to 25 years compared to 2.12% among those aged 26 to 44 years) [461]. Past-year cannabis use in pregnant women was even higher, reaching 11.63% in 2014 [461]. Non-pregnant women counterpart prevalence was higher compared to pregnant, reaching 9.27% for past-month use and 15.93% for past-year use in 2014 [461]. Illicit drug abuse during pregnancy negatively correlated with age, rising to 18.3% amongst pregnant women aged 15-17 [462].

1.3.5.2 Complications: THC is not alone

Cannabis sativa is interestingly unique in its ability to synthesize a vast family of cannabinoids. THC, though exhibiting the greatest psycoactivity, is one of 70 other compounds identified in cannabis plant [459]. The very different pharmacology of CBD results in a non-psychoactive action [463]. Due to CBD antipsychotic properties [464], THC/CBD ratio affects the overall psychoactive potential, leading to high side effects if cannabis contains high levels of THC over CBD [465-467]. THC and CBD content were recently compared between Netherlands, England and USA between 2003 and 2005, highlighting high variability of THC%/CBD% depending mainly on the commercial form (Netherlands: sinsemilla 20.4%/0.2%, herbal 7.0%/0.2%, resin 18.2%/8.1%; England: sinsemilla 14.0%/0.1%, herbal 2.1%/0.1%, resin 3.5%/4.2%; USA: sinsemilla 11.2%/0.2%, herbal 5.0%/0.5% and resin 9.2%/3.9%)[459]. These numbers represent a noticeable change compared to THC/CBD ratios in traditional Moroccan plants used to make traditional hashish (resin) which are closer to 2:1 and resin from countries such as Nepal and Kashmir, having an approximately equal THC/CBD ratio [459]. UNODC's World drug report highlighted increasing concerns over the rising popularity of potent CBD-free high-THC sinsemilla [466], obtained by allowing only females plants to blossom.

1.3.5.3 Synthetic cannabinoids and Smart Drugs: a dangerous new fashion

Synthetic cannabinoids, often referred as "legal high" or "smart drugs", are a large group of new psychoactive substances, available on the market since the mid-2000s, that mimic THC effects and bind to cannabinoid receptors [468]. Damiana (*Turnera diffusa*) and Lamiaceae herbs such as *Melissa*, *Mentha* and *Thymus* are commonly used as an herbal base for the smoking mixtures, on which the synthetic cannabinoids are mixed or sprayed [468]. The first synthetic cannabinoid JWH-018, sold under the brand name "Spice", was detected in Germany and Austria in 2008 [459]. The number of detected synthetic cannabinoids on the market by December 2015 increased to 160 [468]. "Legal highs" thus represent a popular new fashion, characterized by limited data on use prevalence [469] and no available data on long and short term risks [470]. The US Monitoring the Future survey of students suggested 5.8 % synthetic usage prevalence in 2014 for 17-18 years old [468]. Similar percentages were reported by a German study on smoking mixtures and "Spice" among students aged 15–18 in Frankfurt [468]. Concerns on harms related to synthetic cannabinoids are mainly due to the unknown possible side effects, alternative mechanisms of action and manufacturing processes [468]. A peculiar feature of synthetic cannabinoids is their ability to cause mass poisoning. As an example, the cannabinoid Fubinaca was linked to more than 600 poisonings

in Russia in 2014, resulting in 15 deaths; 200 hospital emergencies reported in Poland in 2015 within less than a week; and 33 persons hospitalized in New York City in one night in 2016 [468, 471].

1.3.5.4 Prenatal cannabis: evidence from human longitudinal studies

Richardson and colleagues propose a "double hit hypothesis" for prenatal marijuana exposure [472], with maternal cannabis abuse delivering a "first hit" causing an eCB imbalance in the foetal brain, compromising neuronal network establishment in a way that a "second hit" may cause specific phenotype development [473]. The surprisingly high cannabis usage prevalence amongst pregnant women, together with the increasing THC strength in market compounds and the rapidly increasing availability of synthetic cannabinoids, raises the urgency to address the consequences of prenatal exposure to cannabis [473].

Though the molecular mechanisms of prenatal cannabis are not clear, clinical observations from three prospective longitudinal human studies have been recently reviewed [473]. The Ottawa Prenatal Prospective Study (OPPS) was initiated in 1970 by Fried and colleagues [474]. It included 300 white, low-risk middle class women who self-reported at least six cannabis joints a week before and during pregnancy [474]. OPPS offspring were followed up to 22 years old [475, 476]. The homogeneity of the OPPS cohort study might be taken both as an advantage, being a consistent low variability group with high cannabis use, as well as a disadvantage since the study focused on a low-risk population with limited stressing factors and genetic variance, and thus limited potential for any eventual "second hit" in offspring later in life. The OPPS study reported decreased birth weight, increased startles and tremors and reduced habituation to light 48h after birth [474]. By the age of 3-4 years cannabis exposure correlated with impaired verbal and memory functioning [477]. Externalizing behaviours (i.e. hyperactivity, inattention and impulsive symptoms) significantly increased in cannabis exposed children at the age of 6 [478]. Additionally, by the age of 6 the OPPS study reported an impaired problem-solving ability in situations that required integration of visuoperceptual skills [478]. 18-22 year old young adults confirmed long lasting deficits during visuospatial working memory tasks [475], decreased response inhibition and impaired executive functioning assessed by fMRI [476] in cannabis exposed subjects.

In 1982 the Maternal Health Practices and Child Development Study (MHPCD) was initiated [479]. The MHPCD study focused on high-risk woman, including over 500 mixed-race, mostly single, low socio-economic status woman [479]. The MHPCD cohort was divided by marijuana use during pregnancy into light (less than 3 joints per week), moderate (3 to 7 joints per week) and heavy users (more than one joint per day) [474], with offspring followed up until 14 years old. MHPCD neonates presented an altered EEG recording during sleep [479]. Additionally, growth, sensory/perceptual responses, memory and learning parameters were affected within 1 year of age [479]. The MHPCD study confirmed the results obtained by the OPPS cohort, reinforcing the idea of impaired memory function and decreased verbal scores by the age of 3-4 years, adding an increased tendency towards anxiety and depressive-related behaviours in cannabis exposed offspring [480]. By the age of 10 the MHPCD

offspring cohort exhibited significant impairments in abstract and visual reasoning [481], together with an increased incidence of depression, impulsivity and delinquency [481, 482].

More recently Hofman and colleagues started the Generation R study, a large scale longitudinal study initiated in 2001 that is still ongoing, aiming to follow up more than 10,000 multi-ethnic urban children from foetal development until adulthood [483]. The Generation R study is therefore the first longitudinal study to include data on prenatal conditions for offspring born from cannabis users. Numerous significant impairments were reported in the cannabis exposed foetuses, including reduced growth and head circumference, placental resistance, increased pulsatility and resistance index of the uterine artery, possibly underlying the decreased weight and length of neonates [484]. The impairments reported at 3 years by OPPS and MHPC were not measured in the Generation R study which instead reported increased aggression and inattention at this age in girls [483].

Even if Generation R results on young adults are not available yet, numerous considerations have to be made when comparing the above studies. Cohort sampling was variable within the different studies, including socio-economical and ethnical heterogeneity and cannabis assumption quantities [472, 473]. Moreover, while OPPS and MHPCD studies took place from 1970s to 2000s, the Generation R study involves cannabis products available later in the market, with a likely increase in strength and THC/CBD ratio [472]. These inevitable complications can be simplified by future research in animal models where species and amount of exogenous cannabinoids can be controlled, in order to dissect networks responsible for the cognitive impairments highlighted by human studies.

1.3.5.5 Molecular mechanisms of transgenerational cannabis exposure

The CB_1R is the major target of THC [247], with potential minor signalling contributions through CB_2Rs [485] and GPR55 [486]. Importantly, THC cannot be degraded by endogenous MAGL, or any other endocannabinoid-related catabolic enzyme known to date. Thus, introduction of THC to developing foetal circuits could result in ectopic CB_1R engagement, leading to unwanted directional neurite outgrowth and synapse formation errors [487]. Synthetic cannabinoid studies suggest that chronic cannabinoid agonist exposure causes CB_1R down-regulation and rapid desensitization in a regionally distinct manner [488]. In sum, *in utero* THC is potentially able to reshape endocannabinoid signalling by directly affecting receptors and enzyme levels.

Most transgenerational studies on animal models aiming to elucidate molecular mechanisms of cannabis in the developing brain uniquely analysed the effect of THC administration. However, one of the first studies on perinatal cannabis addressed α1-adrenergic and D2-dopaminergic receptors in the offspring brain upon perinatal administration of both THC and CBD separately [489]. Several genes were found to be significantly altered in succeeding studies. Opioid peptide precursors, including prodynorphin, POMC gene expression and proenkephalin mRNA were altered in several brain regions of rat foetuses prenatally exposed to THC [490]. Neural adhesion molecule L1 expression was significantly increased after

prenatal THC exposure in CB_1R^+ axonal tracts in fimbria, stria terminalis, stria medullaris and corpus callosum [491]

Prenatal THC exposure precipitated persistent behavioural deficits in the adult offspring. Long-term behavioural effects such as rearing, grooming and sniffing, were altered in the adult offspring of both sexes upon perinatal exposure [492]. The same study showed that THC-exposed males exhibited increased exploratory behaviour in a plus-maze paradigm [492]. Hypothalamus-pituitary-adrenal (HPA) axis activation was also evaluated after prenatal cannabis exposure, revealing higher hypothalamic levels of both corticotropin releasing factor (CRF-41) and corticosterone in females [492]. THC-induced dimorphic motor deficit was observed in a later study that proposed increased presynaptic dopamine D2 receptor sensitivity as an underlying cellular mechanism [493]. Prenatal cannabis exposure increases heroin seeking with allostatic changes in limbic enkephalins in adulthood. [494]. Interesting results are provided by a recent study that combined conditional CB_1R strategies and prenatal exposure to cannabinoids. Using this strategy the investigators confirmed that THC-induced long-lasting consequences in adult offspring are solely mediated by its ability to disrupt CB_1R signalling during neurodevelopment [495].

Pioneering a series of studies examining cell subtype selective involvement in mediating prenatal THC exposure deficits, the Fernandez-Ruiz laboratory described altered tyrosine hydroxylase gene expression and activity during early brain development [496] followed by postnatal impairment of tyrosine hydroxylase-containing neurons in rats [497]. Prenatal cannabis-mediated impairment was not limited to neuronal cells, but also described in astrocytes and Bergmann glial cells in the rat cerebellum. Prenatal THC caused deleterious effects on astroglial maturation, resulting in reduced glial fibrillary acidic protein and glutamine synthetase expression [498].

The first effort to correlate cell type specific impairments and long-lasting behavioural deficits provided correlative evidence of enduring cognitive deficits, cortical gene expression and neurotransmission in rats, reporting long lasting THC-induced alterations especially on glutamatergic and noradrenergic systems [499]. The most interesting evidence of a cell type specific engagement upon prenatal cannabis exposure was recently reported by Vargish and colleagues [500]. Maternal treatment with THC induced a significant reduction of Cck interneurons in the offspring hippocampus [500]. Furthermore, residual Cck interneurons displayed decreased dendritic complexity and compromised feedforward and feedback inhibition, possibly responsible for long-lasting impaired social behaviour in animals prenatally treated with THC [500].

Summarizing, eCBs play major roles in neural development and networks establishment. CB_1R, as well as eCB enzymatic machinery expression and subcellular compartmentalization, were shown to be fundamental for neuronal pathfinding and major axonal tracts development. *In utero* genetic and pharmacological eCB manipulation indeed perturbs axonal targeting, potentially leading to long-term networks impairment. Numerous studies confirmed that supra-physiological levels of 2-AG during development and *in utero* exposure to THC, are both able to affect protein and gene expression, impair the development of specific subpopulation of neurons and induce long-lasting behavioural deficits in the offspring. However, molecular mediators of eCBs crucial role in neuronal development remain largely undescribed.

2 AIM

The overall aim of this work was to investigate molecular determinants of endocannabinoid-mediated brain development. Major effort was invested in detailing CB_1R-mediated growth cone extension and eCB roles in the development of axonal bundles and network assembly.

Study I aimed to interrogate upstream signals and molecular effectors of eCB machinery within foetal cholinergic projection neurons.

Study II aimed to describe the role of eCB signalling in regulating oligodendrocyte-growth cone interactions during axonal bundle extension in the embryonic brain.

Study III aimed to reveal molecular determinants and neuronal responses to *in utero* cannabis exposure and examine long-lasting circuit impairments induced by prenatal THC treatment.

Study IV aimed to generate a comprehensive prenatal characterization of cholecystokinin-containing GABAergic cells, the interneuron population with the highest CB_1R expression and therefore potentially most sensitive to perinatal eCB imbalances.

3 RESULTS & DISCUSSION

3.1 *Study I.* Nerve growth factor scales endocannabinoid availability by regulating MAGL turnover in developing cholinergic neurons

The majority of studies on eCB contributions to axonal guidance have focused on the neocortex [234, 375, 376], leaving potential roles in subcortical territories uncharted. Subcortical cholinergic projections to the neocortex express CB_1Rs [268] and release acetylcholine upon CB_1R agonist stimulation [193]. NGF promotes cholinergic neurons survival in the basal forebrain [188], synaptic connectivity [501] and rescues neurotransmission in diseased cholinergic neurons [502]. Adult cholinergic neurons of the basal forebrain where shown to express CB_1R [268] and release acetylcholine in eCB-sensitive manner [193]. However, a potential coordinated action of NGF and endocannabinoids in promoting cholinergic axonal development has not previously been investigated.

3.1.1 Endocannabinoid-mediated axonal development in foetal cholinergic projections

In *study I* we detailed the expression of CB_1R mRNA in subcortical territories at early embryonic ages by *in situ* hybridization. Medial septum (MS) was remarkably enriched for CB_1R mRNA from E14.5 until birth. At E18.5 genetically tagged $ChAT^+$ neurons highly expressed CB_1R protein in cell bodies and along leading processes within the MS, the developing hippocampus and the corpus callosum.

Committing to a systematic *in vitro* study, we detailed the differential localization of 2-AG enzymatic components within foetal cholinergic neurons. While MAGL expression was restricted along the $VAChT^+$ primary neurite, CB_1Rs and DGLα/β were identified at the distal dendrites and in the growth cones, suggesting cell-autonomous 2-AG signalling. In order to explore the functional role of CB_1Rs in cholinergic neuron maturation, we assessed cell fate and morphology upon genetic and pharmacological CB_1R manipulation.

$CB_1R^{-/-}$ and AM251-treated foetuses (3mg/kg, i.p.) both presented an unusual ectopic localization of low-affinity $p75^{NTR}$ in cholinergic neurons of the dorsolateral striatum at E18.5. Sholl analysis of septal $VAChT^+$ neurites at the same age revealed an increased neurite complexity in $CB_1R^{-/-}$ and AM251-exposed foetuses. CB_1R loss of function was therefore sufficient to disrupt neuronal morphology of $ChAT^+$ neurons during development, a possible *primum mobile* of the increased number of striatal cholinergic neurons in $CB_1R^{-/-}$ adult mice. In order to probe for a potential permanent misrouting of long-range cholinergic projections in $CB_1R^{-/-}$ mice, we examined the integrity of the septohippocampal $ChAT^+$ pathway in CA1. $CB_1R^{-/-}$ mice exhibited a significant reduction of cholinergic processes within the pyramidal layer and an overall decrease of MAGL immunoreactive putative presynaptic compartments in the same region of the hippocampus.

3.1.2 NGF regulates cholinergic neurite outgrowth, compartmentalizing 2-AG degradation through sequential regulation of BRCA1 and MAGL

In order to test our hypothesis of a coordinated action of NGF and endocannabinoids on cholinergic neuron development, we coincidently targeted CB_1Rs and NGF high affinity receptors, TrkAs, in neuronal cultures. NGF alone was able to trigger neurite outgrowth and

induce the formation of multiple secondary VAChT^{+} processes. However, if combined with O-2050, a silent CB_1R antagonist, NGF was unable to drive increased neurite development. Thus, NGF-induced neurite outgrowth requires CB_1R activation.

NGF treatment *in vitro* induced a redistribution of the 2-AG enzymatic machinery along cholinergic neurites, resulting in an overall up-regulation of CB_1Rs, DGLα, and MAGL proteins along with increased 2-AG concentrations. Interestingly, the pharmacological inhibition of TrkA signalling significantly reduced neurite outgrowth and redistributed MAGL towards the growth cone, revealing an NGF-mediated compartmentalization of 2-AG degradation.

NGF-mediated MAGL overexpression was stabilized by proteasomal inhibition through lactacystin. Amongst proteasome ubiquitin ligases, breast cancer 1 susceptibility protein (BRCA1) was particularly attractive: BRCA1 was highly expressed during development in ChAT^{+} neurons in the basal forebrain and preferentially located at the leading processes of cholinergic neurons. Proceeding with a subcellular analysis, we confirmed a close apposition of BRCA1 and MAGL in motile neurites and growth cones, suggesting a possible direct molecular interaction. Our hypothesis was confirmed by exposing basal forebrain neurons to cisplatin, a platinum-based anticancer drug able to functionally inhibit BRCA1 activity. Cisplatin application on NGF-treated cholinergic neurons *in vitro* was sufficient to inhibit neuronal outgrowth and MAGL compartmentalization.

Summarizing, in *study I* we reveal NGF signalling at TrkA receptor as a domain-specific molecular regulator of axonal outgrowth, *via* MAGL segregation in cholinergic neurons.

3.2 *Study II.* Endocannabinoids modulate cortical development by configuring Slit2/Robo1 signalling

CB_1R and eCB enzymatic machinery distribution are fundamental for axonal fasciculation and a proper development of major axonal tracts. However, axonal pathfinding is one of the most delicate processes during brain development requiring exquisite coordination of a plethora of timed events [374, 384, 387] including the expression of specific guidance proteins. Amongst chemorepellant molecules mediating neuronal migration, Slit/Robo signalling represents a major phylogenetically conserved mechanism [194]. Although previous studies emphasized a $Robo^{-/-}$ commissural tract phenotype [196] and $Slit1^{-/-}/Slit2^{-/-}$ comparable corticothalamic-targeting defects [202], possible interactions between eCB and Slit/Robo machinery remain unexplored.

3.2.1 JZL184 enhances 2-AG signalling in the foetal forebrain inducing an axonal fasciculation phenotype *via* MAGL inhibition

Aiming to selectively enhance endogenous 2-AG signalling, we administered JZL184 (MAGL inhibitor, 40 mg/kg i.p.) to pregnant mice form E12.5 to E18.5. While other eCB levels remained unaffected by JZL184 administration, 2-AG content significantly increased in mothers and foetal brains. Pharmacological MAGL inhibition by JZL184 exposure was sufficient to provoke an enlargement of L1 neuronal cell adhesion molecule (L1-NCAM)-expressing axonal fascicles, regardless of their projection target, leading to fasciculation of the corticothalamic/thalamocortical axons and significantly increased axonal spread in the corpus callosum. Immunohistochemistry revealed that these axonal phenotypes correlated with altered distributions of radial glial cell marker-2 (RC2) and brain1 $(Brn1)^+$ pyramidal cells. Moreover Neurocan, a proteoglycan essential for cell adhesion [503], was excluded from thickened axonal fascicles in JZL184 exposed brains. A potential confounding influence of CB_1R desensitization was excluded by acute *in vitro* experiments on cortical neurons pretreated with JZL184 for 4 days. Hence, the supra-physiological 2-AG levels likely promoted CB_1R-mediated axonal fasciculation.

3.2.2 CB_1R^+ misrouted axons accumulate Robo1 and host excessive end-feet proliferation of CB_2R-expressing oligodendrocytes

Oligodendrocytes are highly present within the corpus callosum bundle and are postnatally crucial for myelination [504]. Though the expression of CB_1Rs and CB_2Rs in oligodendrocytes has been described [371, 372], their functional role in eCB-mediated neurodevelopment has not yet been investigated. Upon JZL184 exposure, we observed a premature differentiation of oligodendrocytes amongst axonal bundles, confirmed by an increased number of their 2',3'-cyclic-nucleotide 3'-phospodiesterase $(CNPase)^+$ end-feet apposing neighbouring axons. This JZL184-induced premature end-feet proliferation phenotype was observed in both WT and $CB_1R^{-/-}$ axonal bundles, suggesting a mechanism involving CB_2Rs within oligodendrocytes.

3.2.3 JZL184 induces growth cone chemorepulsion *via* Slit/Robo signalling.

Analysing sections from human foetuses at gestational week 20-23, we highlight the co-expression of CB_1R and Robo1 within the same axons in the internal capsule. Upon JZL184 *in vitro* treatment, cortical neurons showed increased expression of Robo1 in the growth cone, in an O-2050 sensitive manner, confirming CB_1R involvement. Robo1 accumulation within the growth cone was confirmed both pharmacologically on synaptosomes and by MAGL acute silencing *in vitro*.

Pathophysiological significance of Robo1 accumulation in the growth cone upon supra-physiological 2-AG levels was notably validated in human foetal subjects *in utero* exposed to cannabis. An increased Robo1 receptor mRNA was quantified in cannabis-exposed subjects, relative to controls. Remarkably, mRNA analysis of the human foetal cortex highlights a significant correlation between the expression of Robo1 mRNA and Slit1 regardless of cannabis exposure, furthermore suggesting a Robo/Slit functional interaction. Human results therefore reinforced our hypothesis that oligodendrocytes prematurely differentiate upon excessive 2-AG exposure, mediated by an overexpression of Slit at the end-feet in a CB_2R-dependent manner. Using oligodendrocytes *in vitro* as a model, we confirmed oligodendrocytes as a main source of Slit2. Hence, upon JZL184 exposure, oligodendrocytes recapitulated the *in vivo* phenotype, showing premature differentiation, and increased submembranous expression of Slit2 that accumulated at the end feet. Neuron-oligodendrocyte co-culture experiments revealed that upon overexpression of Slit2 by oligodendrocytes, nearby neurites increased distance to apposing glial surface in a Robo1-dependent manner. The JZL184 effect on oligodendrocytes was reversed by AM630, a CB_2R antagonist, further supporting an "axonal pathfinding bipartite model" in which oligodendrocytes turn into active players, able to sense eCBs through CB_2Rs and actively interact with Robo1-expressing growth cones. In sum, our findings from *study II* identified 2-AG as an upstream signal molecule able to recruit Slit/Robo interactions in the process of axonal pathfinding (**Figure 8**).

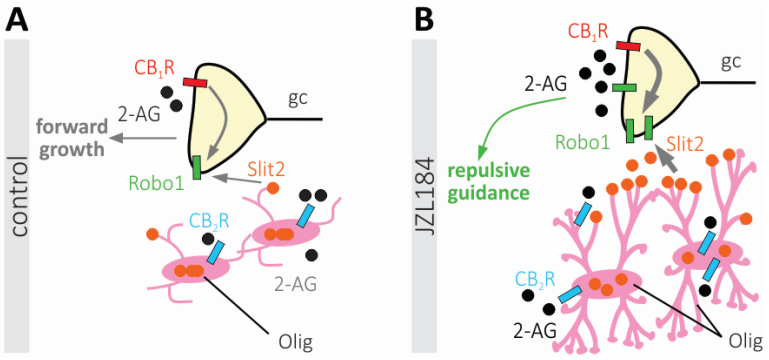

Figure 8. e modulates growth cone behaviour *via* Slit2/Robo1 signalling. **(A)** Schematic overview of eCB-mediated neurite outgrowth *via* glia-growth cone interaction. **(B)** Supra-physiological levels of 2-AG modulate growth cone directionality configuring the downstream Slit2/Robo1 interaction. Adapted from *Study II*, figure 8 [505].

3.3 *Study III.* THC exposure impairs cortical development *via* SCG10 degradation

The startling incidence of cannabis use during pregnancy, which is likely to be exacerbated by recent depenalization policies around the world, demands further investigation into the cell, molecular, and circuit consequences of *in utero* exogenous cannabinoid exposure on foetal nervous system development. To investigate the molecular details of the signalling cascades involved upon maternal THC exposure during pregnancy we first had to establish an accurate mouse model. We administered THC daily at 3 mg/Kg (i.p., from E5.5 to E17.5) [506] which did not affect maternal behaviour, bodyweight, offspring size or sex ratio indicating that we were below threshold for intoxication that can occur with high doses [507]. Male foetuses where probed before birth (E18.5) using anatomical and molecular assays. Postnatal anatomy (postnatal day (P)-10 and P120) and electrophysiology (P120) were examined to probe for any potential long-lasting developmental deficits.

3.3.1 THC affects axonal development and cortical network establishment

THC exposed offspring showed significantly decreased CB_1R^+ perisomatic inputs within the superficial layer I/II of the cortex at both P10 and P120. This observation is similar to findings in interneuron-specific $CB_1R^{-/-}$ mice [376] and is also consistent with a recent study showing significant loss of CB_1R-expressing Cck-interneurons in the offspring of THC exposed mothers [508]. In addition, THC treated offspring displayed long-term functional modifications of hippocampal circuitry with altered synaptic activity at Shaffer collateral inputs to CA1.

THC-exposed foetuses analysed at E18.5 displayed an impairment of axonal bundle formation in the corticofugal system, resembling the well-characterized phenotype of $CB_1R^{-/-}$ mice [374]. THC significantly increased the diameter of the first order fascicles compared to vehicle controls. Administration of AM251 (5 mg/kg; CB_1R antagonist), but not WIN55,212-2 (5 mg/kg; CB_1R agonist) showed a similar phenotype, suggesting CB_1R signalling impairment is responsible of the corticofugal tract fasciculation phenotype. mRNA and protein analyses confirmed involvement of the eCB system, showing reduced CB_1R levels and increased MAGL expression in the embryonic cortex upon THC exposure.

3.3.2 Unbiased proteomics reveals THC-mediated reduction of SCG10 levels

In order to identify the molecular effectors of THC-induced alterations we performed quantitative proteomics on cortices from male foetuses at E18.5 through isobaric tagging for relative and absolute quantification (iTRAQ). Peptides were analysed both by LC-MALDI/MS/MS and nLC-ESI/MS/MS mass spectrometry to profile THC-sensitive proteins. Out of 837 identified hits, the expression levels of 35 proteins changed significantly upon THC treatment. Amongst differentially expressed proteins, SCG10/stathmin-2, a microtubule-binding protein in axons, was exceptionally appealing given that SCG10 is highly expressed during corticogenesis and co-labels with CB_1Rs in long-range projections and growth-cone structures. Proteomic analysis revealed that SCG10 levels were reduced upon THC and these findings were confirmed at both protein and mRNA levels.

3.3.3 THC induces axonal SCG10 degradation *via* proteasome, induced by JNK1

To dissect the molecular cascades underling SCG10 degradation *in vivo* upon THC maternal administration, we availed of both *in vitro* and *ex vivo* models. Our *in vitro* data revealed that THC rapidly induces JNK and Erk1/2 phosphorylation in a CB_1R-dependend manner, driving SCG10 proteasomal degradation in cultured cortical neurons. In order to confirm THC-induced SCG10 degradation within an intact corticofugal projection system, we established an *ex vivo* model of our *in vivo* experimental protocol, using 300 µm organotypic slices from E14.5 forebrains to test the effects of THC treatment (10 µm, 30 minutes) on axonal SCG10 distribution. Acute THC treatment was sufficient to diminish SCG10 expression in organotypic slices as measured by both SCG10 fluorescence intensity and spatial extent. SCG10 acute degradation in corticofugal axons was significantly attenuated by applying SP600125 (JNK inhibitor).

To ascertain the relevance of our findings to the human nervous system, we probed SCG10 expression in foetal human hippocampi from of electively aborted second trimester foetuses exposed to cannabis and compared them to age-matched controls. Hippocampal SCG10 mRNA and protein expression was significantly reduced in cannabis-exposed subjects.

Altogether, in *study III* we identified SCG10 as a molecular effector acting downstream from THC activation of CB_1Rs that leads to long-lasting synaptic miswiring during foetal corticogenesis. Raising further concerns about marijuana consumption during gestation, findings from a recent study show that THC-exposed offspring exhibit persistent loss of Cck^+ interneurons accompanied by deficits in feedforward and feedback inhibition in CA1 and altered social behaviour [500]. Cumulatively, recent findings reinforce caution against even mild cannabis use during pregnancy.

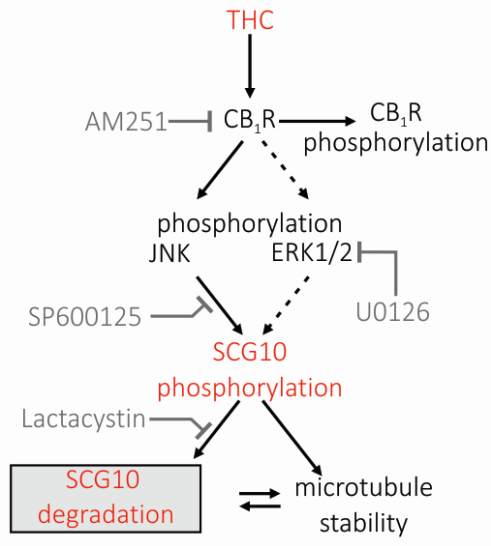

Figure 9. Schematic representation of THC downstream pathway highlighted in *Study III*. *In utero* THC exposure engages CB_1Rs and induces sequential phosporilation of JNK1. This results in SCG10 phosphorylation and degradation *via* proteasome. Adapted from *Study III*, figure 5 [509]

3.4 *Study IV.* Prenatal portrait of cholecystokinin-containing interneurons

CB_1R-expressing Cck-containing GABAergic interneurons [350, 510] were identified as an early neuronal population originating in the CGE [350, 511]. As shown recently by Vargish and colleagues [500], developmental anomalies driven by eCB (or phytocannabinoid) imbalances, chiefly affect Cck-CB_1R-expressing interneurons. Prenatal characterization of Cck^+ interneurons is however limited, mainly due to difficulties in visualizing them histochemically. Máté and colleagues recently developed a new transgenic mouse strain that expresses the T3 variant of the Discosoma Red Fluorescence Protein under control of the Cck promoter (DsRedT3/Cck) [512]. Taking advantage if this mouse line, we examined the spatiotemporal profile and physiological maturation of Cck-expressing interneurons during foetal brain development.

3.4.1 Methodological considerations: cell-specific transgene expression in the DsRedT3/Cck mouse brain

Aiming to characterize the developmental trajectory of Cck^+ interneurons we examined $Cck^{BAC/DsRed}$ cells at E12.5, E14.5, E16.5/E18.5 and P20 focusing on: i) distribution in the whole brain by lightsheet microscopy; ii) molecular identities using immunofluorescence; and iii) cell morphology and excitability using patch-clamp electrophysiology with dye filling of individual cells. Mouse line Tg1 (~1 copy insertion of $Cck^{BAC/DsRed}$) and mouse lineTg47 (additional insertion of more copies ~7) [512] presented differences in fluorescence intensity, but displayed comparable cell distributions resembling previously described Cck^+ cell distribution patterns [513, 514], including that described in the Allen Brain institute database mRNA map. In order to confirm the selectivity of DsRed expression in the DsRedT3/Cck adult mouse brain, we used a combination of *in situ hybridization* and immunohistochemistry. The vast majority of the DsRedT3 transgene-expressing cells co-localized with Cck mRNA. In order to selectively visualize GABAergic/$DsRed^+$ cells, we generated a dual reporter mouse line co-expressing DsRed in Cck^+ neurons and GFP in GABAergic interneurons ($Cck^{BAC/DsRed}$::$GAD67^{gfp/+}$).

3.4.2 Migratory pathway of $DsRed^+/CR^+/CB_1R^+$ interneurons in the forebrain

Despite numerous postnatal studies on Cck interneurons, the ontogeny of Cck expression in the brain during gestation is poorly understood. Early prenatal Cck expression was observed previously in a subpopulation of neural tube and neural crest cells in mouse embryos (E8.5–E9.5) [515], in olfactory sensory neurons [516], and in the thalamus and spinal cord at E12.5 [514]. However, the *in situ* approach used in those studies to identify Cck-expressing cells, precludes additional levels of characterization, such as physiological interrogation or cell migration assays. Using the DsRedT3/Cck line, we developed a spatiotemporal map of Cck-positive cell distributions at E10.5, E12.5, E14.5 and E18.5. Moreover, a modified Cubic-2 clearing protocol [517] combined with light sheet microscopy was used as an innovative and powerful tool to visualize all the originating niches of Cck-expressing cells in the intact brain. The first niche of Cck^+ interneurons leaving the CGE was visualized at E12.5 migrating along the tangential migratory stream (TMS). A large portion of those migrating Cck^+ neurons showed coexpression of Calretinin (CR) (19.5 ± 1.2 %) at E12.5, similar to the 10-30% co-

localization expected in the adult [518]. By E14.5 the majority of DsRed$^+$/GFP$^+$ interneurons migrated along the TMS and reached the superficial layers of the hippocampus. The early biochemical commitment of hippocampal Cck$^+$ interneurons was confirmed by consistent co-expression of DsRed/CR/CB_1R by E14.5. By E18.5 Cck$^+$/GAD67$^+$/CR$^+$ interneuron lamination largely resembled that reported for mature tissue, settling in upper layers II/III of the cortex and SR/SLM of the hippocampus.

3.4.3 Electrophysiological characterization of migrating Cck-positive interneurons

In order to evaluate functional development, patch-clamp electrophysiology was used to determine passive membrane properties, excitability and ion channel expression. For prenatal time points we recorded cells at the pallio-subpallial boundary of the CGE (E12.5), within the tangential migratory stream (E14.5) and in the hippocampus SR/SLM region (E18.5). For postnatal recordings (P16-P20) we recorded in SR of dorsal hippocampus. The excitability of Cck-positive CGE-derived cells was measured in current-clamp mode and compared across different prenatal ages. Developmental K^+ current profiles were evaluated using voltage-clamp recordings to pharmacologically dissect TEA-sensitive K^+-currents and 4-AP-sensitive A-type K^+-currents. In short, we observed the first signs of excitability in Cck$^+$ interneurons by E14.5, with a resting membrane potential significantly more hyperpolarized compared to E12.5. 4-AP sensitive A-type K^+ currents were detected by E14.5. In order to evaluate morphological changes during development, Cck$^+$ interneurons were filled with Lucifer-yellow during recordings. We observed a shift from the expected bipolar migratory morphology at E12.5/E14.5 to an elongated soma with increased dendritic complexity by E18.5. Moreover, by E18.5 interneurons exhibited significantly more hyperpolarized resting membrane potentials, reduced input resistances and reliably fired action potentials in the 4-12 Hz range. 4AP-sensitive A-type K^+ currents increased by 1.5-fold at E18.5 comparted to E14.5, with a further increase in the adult, which in our hands, was the most reliable parameter that positively correlated with cell maturity.

Thus, in *study IV* we used a combinatorial approach to selectively target and interrogate Cck$^+$ interneurons throughout development from early prenatal stages. Interneuron subtype-specific malfunction has been associated with numerous psychiatric diseases including schizophrenia, autism and intellectual disabilities [78]. A cell specific approach is necessary to investigate the physiology of specific interneuron cohorts in each of these disorders. Combining DsRedT3/Cck transgenic mice with immunohistochemistry, whole brain imaging and electrophysiology offers a powerful multiparametric approach for selectively characterizing Cck-expressing interneurons from early embryonic stages through development. The live reporting of Cck-expressing cells in DsRedT3/Cck transgenic mice further makes them available for cell migratory assays, calcium imaging, and two-photon imaging for future *in vitro* and *in vivo* experiments in both physiological and neuropathological models.

4 CONCLUSIONS

Endocannabinoids are key regulators of neurite outgrowth in the embryonic brain due to CB_1R localization in the growth cone and a well-orchestrated compartmentalization of eCB machinery along the elongating neurite and across heterogeneous cells. Indeed, non-physiological eCB levels during brain development are sufficient to induce long-lasting neuronal network impairments. Evidence provided in this work highlighted that:

Study I: prenatal CB_1R manipulation permanently reshapes septo-hippocampal cholinergic projections

: NGF binds to TrkA in cholinergic neurites, inducing growth cone-localized MAGL degradation *via* BRCA1, thus cell-autonomously enhancing 2-AG signalling and neurite differentiation

Study II: MAGL inhibition induces supra-physiological 2-AG levels, leading to axonal fasciculation and premature differentiation of oligodendrocytes within axonal bundles

: supra-physiological 2-AG levels induce Slit2 overproduction in CB_2R positive oligodendrocytes and Robo1 import into CB_1R positive growth cones

: supra-physiological 2-AG levels induce growth cone repulsion *via* Slit2/Robo1 interactions

Study III: prenatal exposure to THC permanently alters synaptic connectivity and physiological responses in the adult offspring

: prenatal THC exposure induces SCG10 degradation in the embryonic neocortex of mice and in the foetal human hippocampus

: prenatal THC binds to CB_1Rs and recruits JNK1 to induce SCG10 proteasomal degradation, promoting aberrant growth cone development

Study IV: CGE-derived Cck-expressing interneurons emerge at the palliosubpallial boundary at E12.5, tangentially migrate to reach the hippocampus by E14.5 and exhibit significant anatomical complexity by E18.5

: Cck positive interneurons express TEA-sensitive K^+ currents as early as E12.5 and exhibit mature electrophysiological features such as hyperpolarized resting membrane potentials and sustained action potential firing by E18.5. A-type K^+ currents are detected from E14.5 and further increase in a manner that correlates with the migratory phase and maturity of Cck interneurons.

Axonal pathfinding, projection development, and synaptic targeting are compromised by eCB imbalances. Thus, exogenous inputs with potential to alter the delicate balance within the eCB system should be carefully monitored during pregnancy. Additionally, unveiling molecular determinants of critical events during brain development, is possibly the shortest path to understanding neurodevelopmental disorders such as schizophrenia, autism and intellectual disabilities.

5 ACKNOWLEDGEMENTS

First and foremost thank you, Tibor! You have been supportive since the day of my interview, when you gave me the chance to start this PhD journey under your wing. Ever since, you guided my personal and professional development. You gave me the chance to work with brilliant colleagues, international collaborators and provided the opportunity to be exposed to several academic environments. I am most grateful for how much you broaden my scientific knowledge during these four years. You shaped me to a more independent scientist and a stronger person. Together we celebrated numerous achievements and shared challenging moments, we dissected samples, climbed trees in Sweden and even played volleyball in Vienna (and packed few boxes between the last two)… just few of the countless memories along this journey together.

I would like to express my deepest gratitude to Chris, my advisor on the other side of the Atlantic. This year at NIH (was it really just one year?) was simply wonderful! I stepped in your lab with no physiology knowledge, though you encouraged me to keep going when I felt lost and you appreciated every success we had. It was a rare gift to be under your wise and generous mentorship. Thanks to you, I fell today more confident and ready to step forward. I truly appreciate you decided to fly to Stockholm for my defence, contributing to this unforgettable day. Not least, thank you for the finest whiskey tasting of my entire life.

My sincerest thanks to Professor Simon Butt, for flying from Oxford to be my opponent. Thank you Professor Andrew Irving, Professor Jesper Haeggström and Professor András Csillag for being part of this extraordinary Examination Board, and to Professor Rochellys Diaz Heijtz for being the chairman of my public defence. It is an honour to have to opportunity to discuss this work with you all.

I would like to extend my warmest gratitude to Ken and Erik. You have been my co-supervisors, you taught me every skill I have today including how to emotionally survive challenging times. You have been supportive and understanding, even when I was most confuse (…and I was multiple times most confused about funny looking neurons in our petri, surprising solutions osmolarity, and many more matters, including life in general). Thank you for each suggestion you gave for this thesis and for the inspiring meetings we had during these years, you definitely shaped my future decisions. This journey would have been unquestionably harder without your support. Thank you, Jason. I guess this is the right paragraph to express my gratitude to you. Thank you for mentoring me into the viral injections word, for all the KGluc aliquots I stole and each time you checked the *lambda*. Even if not my supervisor on paper, your knowledge and your advices in the course of last year certainly contributed to my formation and upcoming direction.

I would like to seize this opportunity to acknowledge the many extraordinary advisors I met along my education. Proceeding backwards in time thank you, Elisabbetta and Professor Michela Matteoli for being exceptionally kind during these years. You have been my master degree supervisors and you were the first to support this PhD. You inspired me as scientists, as women and, most importantly, as a combination of the above. I guess I inherited my enthusiasm for neuroscience from you. I wish to express my gratitude to Roberto Spurio, my bachelor supervisor at Unicam, my email-advisor during the following years and companion of most enjoyable philosophical discussions. My curiosity towards the scientific method roots back to your Inorganic Chemistry classes. Last but not least, I wish to thank those who first shared their commitment to critical thinking and analytic analysis. Thank you Professor Stefania Monteverde, my literature teacher at high school, for your books on Giordano Bruno, Jeann D'Arc, George Orwell and William Golding. Thank you Professor Stefano Perticarini, teacher of mathematics at my secondary school, who (successfully) aimed to teach deductive reasoning to a class of 12 year olds.

I cannot thank enough all past and present members of the Harkany group, in Stockholm and in Vienna. Kasia first of all, for the countless happy memories we have together and Vale for sharing science, projects, pipettes and bench space, and for your attempt to teach me patience! You both have been more friends than colleagues, helped me and inspired me in many ways. Giuseppe for introducing me to the lab and to Stcokholm, and for being my Cicerone at Karolinska. Joanne, Gloria, Mateja, Fatima, Johannes, Agoston and Edit, the Viennese crew! I'm glad I had the chance to work with you, we had fantastic moments (and long evenings) together! Alán, for our valuable discussions, Roman, for your passionate opinion in each scientific debate (and for teaching me the whiskey/apple combo). Mingdong, we started and finished this PhD journey together and I am glad we could share each step and worry we had along (and I cannot thank you enough for your precious help in these last weeks!). Alessa, for the most beautiful cryosections, Patrick for your daily assistance with computers and software (and for your terrific barbecue party!), Romana for your generosity (and for leading the *Cooking Ladies* team), Erzsi for each and every precious advice and Elisabeth for being always encouraging. A special acknowledgment to Professor Oleh Hornykiewicz. Meeting you has been a real honour (and once more thank you for that brain slide, I will always bring it with me).

After I joined Chris group, it did not take long to I realize I was surrounded by incredible colleagues. I am glad I had the chance to discuss science with each of you and learn so much during this year. You have been something like my family in the US. Thank you, Ram for each precious discussion we had, Vivek for your scientific enthusiasm and Tylor for your passion for eccentric Italian movies. My warmest thank to Gülcan, for sharing fears and dreams, for

organizing lunches at *Tandoori Nights* and for introducing me to swing dance (and for that madness with that frame!). I am grateful to each member of the *Rock Bottom Fellowship* (later evolved in *The Little Lebowski Urban Achievers*), each night was just great! Steve, *Fellowship* undisputed leader, good friend and hilarious officemate, Jim for being always encouraging (for initiating us into the *Mug Club* and for the *Jameson*), Rachel for being our undeniable MVP, Geoff for your patience with my DSI recordings, Daniel for the countless times you helped me, Calvin for sharing amusing stories about China. A wholehearted acknowledgment to Pam for arranging all the bureaucracy that made this year at NIH possible and to Xiaoqing, for being sort of a mam for each of us and such a generous person in many ways (including feeding us with you delicious cakes!). I am infinitely grateful to Vincent and Lynne from the MIC facility for sharing your expertise and your passion for imaging. You have been enormously supportive many times during this year. Vincent, thank you for your patience, especially during the time I was optimizing cleared tissue imaging and Lynne for each suggestion you gave (and *God save the Nespresso*!).

Beside my lab colleagues, many others contributed to make me feel at home, whichever country I was. Starting from Stockholm, thank you (Her)Mani! You have been my best Swedish/Brazilian friend, the most brilliant lab- and course mate and supreme party companion. I most enjoyed our discussions about developmental neuroscience, about life and love. Thank you Alis, Vyacheslav, Nina, Claire, Igor, Boris, Karol, Kasra, Moriz, Miryam, Paola, Pia, Simone, Alessandro, Sueli, Moriz, Kasper and Staffan. You all made each day at MBB memorable, and each MBBpub, MolNeuroPub and ElphysPub special. Thank you, Naza for your wisdom (I wish I had a biology teacher like you!), Franci (my favourite straightedge ballerina/mathematician), Stefano (for sitting next to me four years ago on that flight) and Debbie, the dreamer of this companionship. You all contributed to make my Swedish memories so special. Moving to the Viennese counterpart, thank you Tugrul for sharing lights an shadows of this PhD life (and the first *Ikea* shopping), Hugo and Nace for your positive vision, Ornella for initiate me to the *Mozzarella di Bufala Exporters*, and all the members of Professor Thomas Klausberger group, I am glad I had the chance to meet each of you and be exposed to your expertize. I wish to thank Mimi for the fantastic dinners we had, Josef and Caroline for being the best landlords on this planet, Ádám and Dr. Áron for coming over from Hungary and Öcsi (that wooden ladder was definitely too heavy!). On the other side of the Atlantic, I cannot thank enough Marta, it was just too good to have another *Marchigiana* on the same floor at NIH (thank you for being my guide to this country, such a great host and for preparing the best *galantina* ever, under *Kandel*'s weight) and Gianvito for not giving up yet on my social skills. I am grateful I had the luck to have Farideh and Hassan as landlord in Bethesda, most kind and always helpful during last year. Thank you Katie, Ian, Sara and Lauren (and Pancake), amusing weekend-flatmates in New York.

And here my words for the first (and oldest) companionship: *Quelli Di Urbisaglia*. È incredibile quanto mi siete stati vicino in questi anni, pur essendo i piu' distanti. Grazie per le birre su Skype ad orari impossibili, per esserci stati sempre e comunque. Grazie perchè potevamo trascurare quest'amicizia logisticamente complicata, ma non l'abbiamo fatto. Perchè *Quelli Di Urbisaglia* son fatti così, non dimenticano, mai! Grazie Gloria, grazie Saretta e grazie Andre. Grazie Patrizio, abbiamo iniziato insieme questo percorso e dai che ce l'abbiam quasi fatta! Probilmente non lo avrei finito questo dottorato se tu avessi smesso di farmi sorridere.

Talking about Italians, I want to conclude thanking my family, the constant source of my life energy. They supported me far beyond their possibilities. Grazie mamma Morena e babbo Giuliano, devo a voi questo dottorato. Per avermi sempre incoraggiato, per esservi fidati, per aver compreso quanto questo dottorato fosse importante e per avermi lasciato andare, nostante quanto sia difficile esser lontani. Per non aver mai dubitato e aver fatto di me la persona che sono oggi. Grazie zia Enrica, per non essere mai stata solo una zia, ma consigliera, compagna e amica. Un grazie tutto speciale a mia nonna, Maria, esempio e roccaforte. Pur non sapendolo, mi sei stata spesso indispensabile in questi quattro anni. Grazie zia Cristina, zio Silvo, Ele, Vale, Adriano e le piccole Giorgia e Ludo per avermi sempre supportato, per quanto insolite fossero le mie scelte. Non potrei desiderare famiglia migliore. Köszönöm Klárának és Jánosnak, hogy feltétel nélküli szeretetükkel megajándékoztak, segítettek az elmúlt években és befogadtak, ezért a magyar családomként tekintek rájuk és mindmáig ámulatba ejt, hogy mennyire kellemes és nagyszerű emberek. Last, my deepest thanks go to you, Janos. You stood by my side, every day of these four years. We were never certain what would be next, yet you always knew it would be together and truly *nothing else matters*.

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